NOV 24 1924 Medical Lib

THIS NUMBER COMPLETES VOLUME IX

VOLUME IX NUMBER 6

JOURNAL OF BACTERIOLOGY

OFFICIAL ORGAN OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

NOVEMBER, 1924

EDITOR-IN-CHIEF
C.-E. A. Winslow

It is characteristic of Science and Progress that they continually open new fields to our vision.—Pasteur.

PUBLISHED BI-MONTHLY
WILLIAMS & WILKINS COMPANY
Mt. Royal and Guilford Aves.
BALTIMORE, U. S. A.

Entered as second-class matter April 17, 1916, at the post-office at Baltimore, Maryland, under the act of March 3, 1879. Acceptance for mailing at special rate of postage provided for in section 1103, Act of October 3, 1917. Authorized on July 13, 1918.

Copyright, 1924, Williams & Wilkins Company

Price, net $5.00 per volume; United States, Mexico, Cuba, Canada postpaid $5.30 per volume, other countries.

Made in United States of America

PEPTON

Perfectly serviceable for the formulas and in all the technic of the bacteriological and autiform laboratory. It is employed in the usual proportions and for whatever purposes Pepton of this most desirable quality is required.

Manufactured by

Fairchild Bros. & Foster

New York

FOR INSULIN RESEARCH

PFANSTIEHL Rare Sugars and Amino Acids are used by the leading investigators of Insulin.

In their work "Glucose, C.P. (Dextrose) and I Mannose are of significance in intravenous injections" see The American Journal of Physiology, Vol. 61, No. 2, "The Influence of Sugars and Other Substances on the Toxic Effects of Insulin," Noble and Macleod).

Pfanstiehl Sugars and Amino Acids are the standard for research.

Write for our booklet of Rare Sugars, Amino Acids, Bi- or Poly-Saccharides and Rare Laboratory Reagents.

SPECIAL CHEMICALS COMPANY

HIGHLAND PARK, ILL.

ACID-FORMING STREPTOCOCCI AS INDICES OF FOMITES POLLUTION[1]

C.-E. A. WINSLOW AND DOROTHEA H. SANJIYAN

Department of Public Health, Yale School of Medicine

Received for publication, May 6, 1924

It is to Gordon (1904) that we owe the original suggestion of employing the acid-forming streptococci as indices of pollution from buccal sources in the same way that colon bacilli are commonly used as an index of pollution from fécal sources. He demonstrated that streptococci are the most abundant microörganisms present in saliva and that the most characteristic type is *Str. brevis*. This specific name was used in general at that time for all short-chained streptococci, but Gordon further limited it to those organisms clotting milk and reducing neutral red. He found the organism, as thus defined, to be present in saliva to the number of 10 to 100 million per cubic centimeter. He detected it in the air in front of persons speaking loudly. In London air on the other hand this lactose-fermenting streptococcus was found in only one out of ten 50 liter samples and on Blackheath it was not found at all in eleven samples totaling 1000 liters.

Later, Andrewes and Horder (1906) reclassified the streptococci and redefined the common salivary type as *Str. salivarius*. Andrewes (1908) in a study of the bacteriology of sewer and drain air reports that of sixty-four streptococci isolated from air by Gordon and himself 65 per cent were of the *Str. equinas* type (which fails to ferment lactose) while only 6 per cent were of the *Str. salivarius type*.

In regard to Gordon's first contention—that the acid-forming streptococci are among the most characteristic microörganisms

[1] Based in part on a thesis submitted for the Certificate in Public Health, Yale University.

of the buccal cavity it is unnecessary to attempt an extensive citation of literature. Goadby (1903) in his text on dental bacteriology drew particular attention to the short chained streptococci which he found in all mouths "healthy or unhealthy, clean or dirty," and which he also found in the mouths of dogs, rabbits, monkeys and guinea-pigs. DeLeon (1904) from the standpoint of surgery and Hamilton (1905) from that of the epidemiology of scarlet fever, showed that streptococci in considerable numbers are discharged in the mouth spray. Hamilton found that streptococci were present on the tonsils of 47 out of 50 cases of scarlet fever, that they were expelled by coughing or crying in 20 out of 34 cases and by expiration in 13 out of 16 patients who were too ill to cough or cry. In a control series of 50 normal persons streptococci were found on the tonsils in 46 cases, on plates held before the mouth when coughing in 33 cases and on plates held before the mouth while speaking in 26 cases.

The use of the streptococcus as a measure of atmospheric pollution has been developed by Nolte (1914) and by Winslow and Browne (1914) with very encouraging results. Nolte found that cocci from saliva were almost always able to coagulate milk and to form acid in lactose and sucrose broth while cocci from the skin and the air rarely gave either of these tests. The examination of one sample of air in an unventilated laboratory showed 1 acid-forming streptococcus in about 2 liters of air. Five samples in a crowded street car gave positive tests three times, the streptococcus being present in one case in the proportion of about 1 in 4 liters, in another in the proportion of 1 in 2 liters and in the third case in the proportion of 100 per liter. Two tests in a well-ventilated vaudeville theater and two tests in a crowded but well-ventilated department store were negative. Four tests made out of doors gave three negative results while in the fourth case 1 acid-forming streptococcus per liter of air was found. Winslow and Browne (1914) give the results cited in table 1 for a much larger series of examinations.

In the examination of samples of dust from various sources Winslow and Kligler (1912) report an average or 22,040 acid-

forming streptococci per gram in 25 samples of indoor dust and 42,500 per gram in 25 samples of street dust. It is of special interest however to note that of the total bacteria in street dust 1 in 1157 was a streptococcus while in house dust the proportion rose to 1 in 215.

In regard to the problem with which we are here particularly concerned—the presence of acid forming streptococci on fomites—there are of course almost innumerable reports of the presence of pathogenic types of these organisms on various objects. Of the studies which have been conducted in a more or less quantitative fashion we may mention the following examples.

TABLE 1

Average microbial content of air (Winslow and Browne, 1914)

SAMPLES		MICROBES PER CUBIC FOOT		STREPTOCOCCI PER CUBIC FOOT
Sources	Number	20°C.	37°C.	
Country	85	56	30	12
City	134	72	32	11
Offices	87	94	80	22
Schools	684	96		30
Factories	47	113	63	43

Kinyoun (1905) in a study of the bacteriology of the railway coach found *Str. pyogenes* twice in the examination of 64 swabs from the interior surfaces of the cars (15 square feet being swabbed in each sample) once in the examination of 96 air samples (of 1500 to 7200 cubic feet each) and once in an examination of 21 drinking cups. He did not look for the non-pathogenic mouth forms. More recently similar examinations have been made by Weaver and Murchie (1919) who report hemolyzing streptococci present on the hands in 16 per cent of 45 samples from the hands of internes, in 9 per cent of 268 samples from the hands of pupil nurses but in only 2 per cent of 51 samples from the hands of specially trained communicable disease nurses. Six per cent of 137 swabs from door knobs showed the same organism.

Cumming (1919) gives the figures tabulated below as the result of a series of examinations for the presence of *Str. hemolyticus* and *Str. viridans*.

OBJECTS TESTED	NUMBER OF TESTS	PER CENT POSITIVE
Str. hemolyticus		
Tableware wash water	17	94
Tableware	23	91
Mess-kit wash water	29	90
Floor dust from ward containing streptococcus cases.	39	75
Hands of streptococcus carriers	340	37
Various objects	29	24
Str. viridans		
Hands of carriers		100
Tableware	2	100
Various objects	15	14

In a similar investigation, Saelhof and Heinekamp (1920) found *Str. hemolyticus* on 6 per cent of a series of 63 glasses, dishes and eating utensils from a group of restaurants in the condition in which these objects were furnished to new customers. Two of the 4 cultures found were isolated from plates and 2 from knives.

Finally, Matousek (1921) reports finding hemolytic streptococci once in 10 samples taken from the wall of a diphtheria ward, three times in 7 cultures from the soles of nurses' shoes, not at all on hands, floor, door knobs, hand brushes and electric light buttons, all these samples being taken in a diphtheria ward. In a scarlet fever ward hemolytic streptococci were found in 6 per cent of 32 samples from the exterior of the face masks worn by nurses, in 4 per cent of 25 hand samples, in 28 per cent of 14 floor cultures, in 2 out of 9 wall cultures, in 3 out of 6 shoe cultures, in 4 out of 11 used dishes and not at all on door knobs or washed dishes.

OBJECTS OF THE PRESENT INVESTIGATION

Our object in the present investigation was to conduct a considerably more extensive investigation than has heretofore

been reported in regard to the abundance of acid-forming streptococci upon objects of various sorts in order to see what relationship exists between their presence and the degree of salivary pollution to which such objects have been exposed. We have made no attempt to differentiate specific pathogenic types for we desired to find a generally serviceable index of pollution comparable to the colon test for potable water. The presence of specific pathogens on fomites, like that of the typhoid bacillus in water, could be demonstrated too rarely to be of general value. We have therefore used the entire group of lactose fermenting streptococci as our index and have conducted a series of over 1000 different tests in this fashion.

TECHNIQUE OF THE INVESTIGATION

All our samples were collected by the use of special swabs made of pine sticks to the end of which were fastened flat circular discs of wood $\frac{1}{4}$ inch in diameter placed at right angles to the sticks. The discs were covered with pieces of flannel cloth, tied around the sticks just above the disc so as to give a somewhat rough and adhesive surface. The swabs as thus constructed were placed in long tubes or flasks so that the upper ends of the sticks alone protruded. Sufficient moistened filter paper was placed in the bottom of the flask to keep the flannel moist enough to pick up material easily. The flasks were plugged with non-absorbent cotton (fitted around the ends of the sticks) and sterilized in the autoclave.

In taking a sample one swab was removed and the surface to be examined was swabbed in one spot, the swab being firmly applied to the surface and rotated once or twice without being removed from the spot to which it was applied. Each test therefore gives us the bacteria removed by the pressure of moist cloth from a unit surface of the object in question corresponding to a circle $\frac{1}{4}$ inch in diameter.

After taking a sample in this fashion the swab was at once placed in a test tube of lactose broth (standard Bacto medium). On returning to the laboratory the swab was moved up and down several times in the test tube to aid in detaching the organisms

from its surface and then removed. The broth was incubated at 37° for forty-eight hours, special tests indicating that incubation for an additional twenty-four hours did not materially increase the proportion of positive results. At the end of this incubation period the tube was examined and if no growth appeared was discarded. If any growth was present streaks were made on litmus-lactose-agar and streptococci were identified after twenty-four hours at 37° by the appearance of the characteristic pinpoint colonies with marked reddening of the medium. In all the 1923 studies (including four-fifths of all samples examined) the identification was further checked by microscopic examination for cocci occurring in pairs and short chains. This step is really unnecessary however as the characteristic colonies on litmus-lactose-agar are practically never produced by any bacteria except those of the streptococcus group.

In the 1924 series of samples the tests were made in more completely quantitative fashion as follows. After the swab had been removed from the original lactose broth tube, the tube was thoroughly shaken and one cubic centimeter of its contents inoculated into a second broth tube. From the second tube a third was inoculated and so on until we had a series of aliquot dilutions running up to one ten-billionth of the material originally derived from the swab. By this means it was hoped that we could secure a more exact conception of the actual number of streptococci present, as is done in the ordinary examination of water for the colon bacillus.

THE PRESENCE OR ABSENCE OF STREPTOCOCCI ON UNIT SURFACE OF THE DIFFERENT TYPES OF OBJECTS EXAMINED

As a control for our examination of fomites we made 50 swabs directly from the mouth itself, applying the cloth covered disc to a unit surface of the tongue of a series of different individuals. The first tube inoculated from the swab showed acid-forming streptococci in but 27 instances or 54 per cent of the total. As will be pointed out later streptococci were really almost always present as indicated by tests in higher dilution but this figure of 54 per cent is the one to be used in a primary comparison with

our other data. The failure to find streptococci in the first dilution tubes from so many of these tongue samples was presumably due to overgrowth by the many other types of bacteria present.

Our second series of tests were made on objects which had been directly exposed to contamination from the mouth, such as cups, glasses, spoons and forks immediately after use, tooth brushes, telephone receivers, the gummed flaps of used envelopes and the surfaces of drinking fountains. Of a total of 176 such swabs 39 or 22 per cent gave positive results for the acid-forming streptococci.

The next series of samples were representative of objects exposed to contamination from the hands but not directly from the mouth. They included doorknobs, telephone receivers, faucets, panels of swing doors at the points where the hand would

TABLE 2

Presence or absence of acid-forming streptococci on unit surfaces of different classes of objects

CLASS OF OBJECT	NUMBER OF SAMPLES	NUMBER POSITIVE	PER CENT POSITIVE
Tongue	50	27	54
Exposed to mouth contamination	176	39	22
Exposed to hand contamination	609	117	19
Not recently contaminated	269	10	4

naturally be placed, drinking fountain handles, penholders and pencils from public places, hand rails, push buttons, piano keys, the arms of wooden chairs, newspapers, letters and pamphlets recently handled, the keys of typewriters and adding machines, and eating utensils which had been washed and prepared for use. Of 609 samples of this type 117 or 19 per cent were positive.

Finally a series of samples were taken from locations which were not presumably exposed to recent salivary pollution of any sort. They were taken from walls 6 feet or more above the ground, from windows which had not recently been washed, from door frames, from the underside of chairs, desks and tables, from the glass of pictures, from the leaves of books which had not been used for some time, and from walls, fences and other

out-of-door locations at points where hand contamination was highly improbable. Out of 269 samples in this group 10 or 4 per cent were positive.

CLOSER QUANTITATIVE ANALYSIS OF ABUNDANCE OF STREPTOCOCCI FROM VARIOUS UNIT SURFACES

The results as so far analyzed were suggestive of a generally significant relation between the prevalence of mouth streptococci and the extent of salivary pollution but the correspondence was not quite as close as we had expected. We therefore proceeded to a closer analysis of the quantitative distribution of streptococci on particular objects.

TABLE 3

Presence or absence of acid-forming streptococci on different classes of objects on one or more of three adjacent unit areas

CLASS OF OBJECT	NUMBER OF OBJECTS	POSITIVE RESULTS ON							
		1 area only		2 areas only		3 areas		1 or more areas	
		Number	Per cent	Number	Per cent	Number	Per cent	Number	Per cent
Exposed to mouth contamination..	42	11	26	5	12	10	24	26	62
Exposed to hand contamination...	178	43	24	27	15	5	2	75	42
Not recently contaminated.....	73	6	8	1	1			7	10

Of our grand total of tests 879 were made in 1923 and at this period of the study three swabs were taken from each particular object examined at three different points upon its surface. In many instances of course one or two of the swabs from a single object were positive while the other two or the other one proved negative. If we consider not individual unit surfaces but objects we obtain the results indicated in table 3.

The difference here appears rather more striking. Of 73 presumably uncontaminated objects studied only one showed streptococci on more than one of three adjacent areas swabbed and only 10 per cent showed any at all while of 42 objects exposed to mouth contamination over a third showed streptococci on more

than one unit area and 62 per cent showed them on one or more such areas.

Finally in our last studies conducted during the present year (1924) including 228 different sources we made only one swab from each object but sought to determine quantitatively the number of streptococci present upon that particular unit area by the dilution method. This procedure, as perhaps might have been expected, did not prove very satisfactory. It generally happened that one or more dilutions were negative while higher dilutions gave positive results and sometimes a positive result would appear after five or six lower dilutions had proved negative. These irregularities were particularly apparent in

TABLE 4

Approximate number of acid-forming streptococci on different classes of objects as determined by the dilution method

CLASS OF OBJECT	NUMBER OF OBJECTS	NEGATIVE	POSITIVE RESULTS IN FRACTIONS OF ORIGINAL TUBE								
			1	0.1	0.01	0.001	0.0001	0.00001	0.000001	0.0000001	0.00000001
Tongue	50	3	6	2	9	14	9	5	1	0	1
Exposed to mouth contamination	50	14	12	12	4	1	4	1	2		
Exposed to hand contamination	75	48	10	12	5						
Not recently contaminated	50	39	6	1	3	1					

the samples from presumably uncontaminated sources and in the hand contaminated objects and were rare in the samples taken directly from the tongue. Where a considerable time has elapsed since the primary pollution it is natural that isolated mouth organisms should have perished leaving only those which were imbedded in masses of dried saliva. Such masses would naturally be very unevenly distributed and might not be broken up until several successive dilutions and shakings had been made. We dealt with this condition by the process of reversion used in the determination of colon bacilli in the shell liquor of oysters, filling in all negative places below those

which proved positive by transferring downward an equivalent number of positive results. When adjusted in this way we obtained the results presented in table 4 and figure 1.

The results vary enormously as might be expected but a general relation to the presumable extent of contamination is clearly

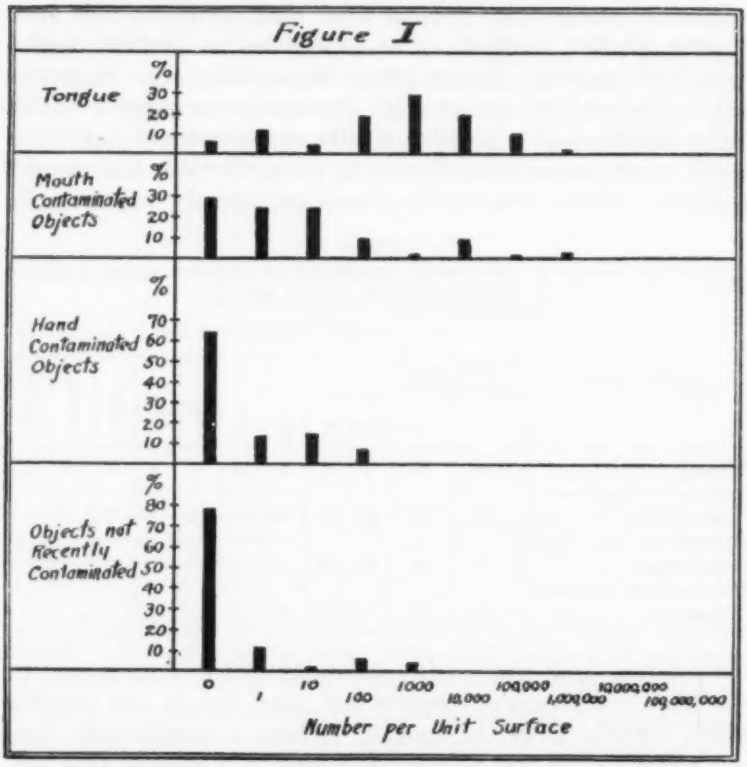

FIG. 1. NUMBER OF ACID-FORMING STREPTOCOCCI ON DIFFERENT CLASSES OF OBJECTS AS DETERMINED BY THE DILUTION METHOD

indicated. On the tongue, acid forming streptococci were found in nearly every case and the mean number present was about 1000 per unit area with a maximum of 100,000,000. On the objects exposed to direct mouth contamination the mean value was about 10 per unit area with a maximum of 1,000,000. The

hand contaminated objects and those from uncontaminated sources on the other hand never gave values in excess of 1000 per unit area while nearly two-thirds of the hand contaminated samples and nearly four-fifths of the presumably uncontaminated sources were negative in all dilutions. It is somewhat disconcerting however to find that when a series of dilutions are made so that clumps are very thoroughly shaken up even as large a proportion as one-fifth of these presumably uncontaminated sources shows positive results. These eleven sources which contained typical salivary streptococci included five samples

TABLE 5

Summary of results

CLASS OF OBJECT	UNIT AREAS POSITIVE IN FIRST DILUTION TESTED		OBJECTS POSITIVE ON ONE OR MORE OF THREE ADJACENT UNIT AREAS		UNIT AREAS POSITIVE WHEN STUDIED IN 10 SUCCESSIVE DILUTIONS		MEAN NUMBER STREPTOCOCCI PER UNIT AREA	
	Number tested	Per cent positive	Number objects	Per cent positive	Number areas	Per cent positive	Number tests	Mean number
Tongue	50	54			50	94	50	1,000
Exposed to mouth contamination	176	22	42	62	50	72	50	10
Exposed to hand contamination	609	19	178	42	75	36	75	<1
Not recently contaminated	269	4	73	10	50	22	50	<1

from the underside of laboratory tables, five from high up on corridor walls in the Sterling Hall of Medicine and one from the roof of a building. None of these areas were at points where the hands would ordinarily go and the organisms in these locations could not possibly be considered as evidence of very recent salivary pollution.

SUMMARY AND CONCLUSIONS

The general results of our study are summarized in table 5.

It seems evident from these results that there is a general relation between the presence of acid-forming streptococci upon

various classes of objects and the extent to which these objects have been exposed to recent salivary pollution. On the surfaces of the mouth these organisms are constantly present in large numbers, although they were absent in 46 per cent of the dilution first tested as a result of overgrowths.

On objects which have been directly exposed to mouth contamination they were found on only one-fifth of all areas swabbed in the first dilution tested but when three adjacent areas were examined 62 per cent of these objects showed positive results. The mean number of streptococci present on all objects of this class was about 10 per unit area (a circle $\frac{1}{4}$ inch in diameter).

On objects exposed to hand contamination 19 per cent of all the areas studied were positive but 42 per cent of the objects of this class showed streptococci when three adjacent areas were swabbed. A series of ten successive dilutions from each area gave 36 per cent of positive results.

It would seem that the swabbing of a single area gives results in the first dilution which may be seriously in error as a result of the effect of overgrowths since 46 per cent of swabs from the tongue examined in this way proved negative. On the other hand the working out of a series of successive dilutions from each swab is accompanied by marked inconsistencies due to irregular distribution and gives 22 per cent positive results on objects not exposed to any recent salivary pollution. The test which corresponds most closely to the actual degree of exposure of the objects tested would appear to be the examination in single dilution of three swabs taken from adjacent areas on the surface of the object in question. By this procedure streptococci were isolated from 62 per cent of objects directly exposed to mouth pollution, from 42 per cent of objects exposed to hand pollution and from 10 per cent of objects not recently contaminated.

The fact that objects quite removed from any probability of recent contamination, such as walls 6 feet above the ground or the undersides of chairs and tables, gave positive results in first dilution, from 4 per cent of the total areas tested and from 10 per cent of all objects tested when three adjacent areas were

examined and in one or more of a series of ten successive dilutions from 22 per cent of the areas examined indicates that groups of these organisms may be found widely distributed on the surfaces of inhabited buildings. This finding sets rather definite limits to the categorical use of the streptococcus as an indicator of recent salivary pollution.

REFERENCES

ANDREWES, F. W. 1908 Report on the microörganisms present in sewer air and the air of drains. Supplement to the XXXVI Annual Report of the Local Government Board, containing the Report of the Medical Officer for 1906-1907, p. 183.

ANDREWES, F. W., AND HORDER, T. J. 1906 A study of the streptococci pathogenic for man. Lancet, **171**, 708.

CUMMING, J. G. 1919 A brief review of indirect contact transmission and a preliminary report of corroborative laboratory research. Amer. Jour. Pub. Health, **9**, 414.

DELEON, M. 1904 Ueber die Gefahren der Wundinfection durch das Sprechen bei Operationen. Archiv für klinische Chirurgie, **72**, 904.

GOADBY, K. M. 1903 The Mycology of the Mouth.

GORDON, M. H. 1904 Report on a bacterial test for estimating pollution of air. Supplement to the XXXII Annual Report of the Local Government Board containing the Report of the Medical Officer for 1902-1903, p. 421.

HAMILTON, A. 1905 Dissemination of streptococci through invisible sputum. Jour. Amer. Med. Assoc., **44**, 1108.

KINYOUN, J. J. 1905 The bacterial content of the railway coach. The Medical News, **87**, 193.

MATOUSEK, W. J. 1921 Contamination of the hands and other objects in the spread of contagious diseases. Jour. Amer. Med. Assoc., **76**, 1490.

NOLTE, A. G. 1914 The identification of the most characteristic salivary organism and its relation to the pollution of air. Ann. Mo. Botan. Gard., **1**, 47.

SAELHOF, C. C., AND HEINEKAMP, W. J. R. 1920 Recovery of *Streptococcus hemolyticus* from restaurant tableware. Amer. Jour. Pub. Health, **10**, 704.

WEAVER, G. H., AND MURCHIE, J. T. 1919 Contamination of the hands and other objects in the spread of diphtheria. Jour. Amer. Med. Assoc., **73**, 1921.

WINSLOW, C.-E. A., AND BROWNE, W. W. 1914 The microbic content of indoor and outdoor air. Monthly Weather Rev., **42**, 452.

WINSLOW, C.-E. A., AND KLIGLER, I. J. 1912 A quantitative study of the bacteria in city dust with special reference to intestinal and buccal forms. Amer. Jour. Pub. Health, **2**, 663.

BARTOLOMEO BIZIO'S LETTER TO THE MOST EMINENT PRIEST, ANGELO BELLANI, CONCERNING THE PHENOMENON OF THE RED-COLORED POLENTA*a

[TRANSLATED FROM THE ITALIAN]

C. P. MERLINO

Hobart College, Geneva, New York

Received for publication May 10, 1924

Three years have already elapsed since the strange reddening of "polenta" (corn meal mush) created such a stir. And as I have ever since been reminded of it by the return of summer, I feel that I should give exact information regarding all the circumstances which accompany that phenomenon and of the special characteristics of this new organism, adding thereto a brief review of the fortunes of those things that I, before any other, made public concerning this subject.

In order not to omit anything strictly pertinent to the topic, I deem it necessary to tell you the date of what may be considered to be the first appearance of the phenomenon. Without a doubt it was first observed in the early part of July, 1819, although I am of the opinion that it has manifested itself at other times in the warm season whenever circumstances conducive to its production have combined. Nor do we need the testimony of peasants to prove this, as Melo[1] thinks. Indeed such testimony would lead us to think the contrary. Why then did they marvel and create such an excitement when they saw on the aforementioned date the surface of the polenta take on a brilliant red color? If, according to Melo, they knew that such a reddening takes place in warm, rainy seasons, it follows that it would have been

*Letters are used to designate translator's notes placed at the end of the article. Figures are used for the footnotes that appear in the original.

[1] See Giornale dell'italiana letteratura del Da Rio, tom. 49, pag. 333.

so well known that the people could have had ample time to study the conditions which lead to its production—which study was not made.

I say, nevertheless, that the phenomenon must have appeared at other times, but I conclude this from the results of the experiments which indicated the circumstances necessary for its appearance. And if you ask me why the peasants noticed the phenomenon only at the aforementioned time, I shall reply that conditions at that time were such that the effect was very noticeable.[2] In this case, the whole surface of the polenta took on a bright red color; whereas in other cases only small colored spots appeared, unobserved by the ignorant peasants. This, then, I consider to be the reason why the appearance of the phenomenon did not cause a commotion until 1819. Nor will I ever accept the explanation of Melo, who ascribes the production of the phenomenon to the great corn crop of that year. True, the harvest was unusually great that year, but it is none the less true that there was an equal abundance in other years without the production of the phenomenon to such a degree as that of 1819. Furthermore, I have constantly observed that, in addition to a warm and damp atmosphere, the phenomenon is never developed so fully as in 1819, unless the polenta be placed in foul air.

Still insisting that the rich crops were the cause of the phenomenon, Melo assures us that in that year the farmers made such lavish use of the polenta that they always had a surplus, even two or three days after having made it. In calling our attention to this, Melo would have us believe that such a long period is necessary for the formation of the phenomenon. This is not true, since the red color appears within twenty-four to thirty hours, if the atmosphere is damp enough. Indeed, later than this time other types of molds appear which effectively impede the red coloring. This agrees with what is generally

[2] It is a fact that in 1819 the temperature rose higher than it had for several years. This condition no doubt favors a greater evaporation which makes a greater humidity. It would be tedious to discuss all of the effects which may be produced in the atmosphere by an unusually warm summer.

the case; that is, that larger plants so weaken the smaller ones that these latter, sapped in their strength, finally die.

I consider it well to inform you of Melo's error with regard to the time necessary for the production of the phenomenon so that you may see clearly that under favorable conditions it can be produced without a rich harvest, since in many cases it does not require even 24 hours before the polenta becomes tinged with red, as I shall explain further.

It would therefore be of no avail to discuss the date of the first appearance of the phenomenon, for the causes that produce it and the substance on which it manifests itself show conclusively that the phenomenon must have had its origin at the time when corn meal (maize) or the flour of other cereal grains came to be used as food.

Instead, then, of engaging in further research on this point, it is better to state again that the single year 1819 may be considered as the first in which this red growth was observed; and it is better to emphasize it so that Melo may not lead us in error by referring to an observation of the phenomenon some years previous. The clearest proof to convince him of his mistake is to remind him of the stupor and marvel, and even fear, of the peasants who considered the coloring of diabolical origin, calling the phenomenon the "bloody polenta." Indeed their fear was so great that they would not live under the same roof where such supernatural influences were operating. They turned thence to the priests imploring them to banish such maleficent spirits. It even happened that Abbé Melo himself was asked to go to the house of a certain Borgato,[2] a farmer in the district of Padua, to free it from evil spirits. This, of course, is the natural philosophy of the people; that is, to ascribe to supernatural causes all those things of whose origin they are ignorant. And since ignorance not infrequently is the cause of calumny, the families in whose homes the phenomenon occurred, were charged with all sorts of evil doings. To this, then, must be attributed the reason for the commotion caused by the appear-

[2] See Da Rio, op. cit., t.c., pag. c.

ance of the phenomenon; an excitement which reached such proportions as to demand the intercession of the Police Department, who appointed a Commission, composed mainly of professors of the University of Padua, to investigate the phenomenon. This was peculiarly fitting, inasmuch as the greatest stir arose in Paduan territory, and particularly in the town of Legnaro, at the house of a certain Antonio Pittarello.

Several days, however, passed without any information on the origin of the phenomenon before I sent a paper on the subject to the Governmental Authorities, explaining therein that the coloring of the polenta was but a natural effect. That same day, August 22, I informed several educated persons of this city of the results obtained from my experiments. Among these persons, were Professor Innocente, who desired to have some of my colored polenta, and Dr. Dal Negro, a brother of the eminent professor in the University of Padua, and member of the Commission appointed to study the phenomenon. Due to this, the results were divulged rapidly, although I did not publish them in the Official Gazette until the 24th of the same month.

For several days succeeding its publication, there was much discussion of my experiments in the columns of this newspaper; and to make them still more well known, an enterprising publisher printed them in a small pamphlet, which he sold in the streets, so that the general public as well as educated persons came to know about them.

But leaving aside this general discussion, I shall expound that which is strictly pertinent to science; and not wishing to omit anything which may serve to elucidate the causes of the phenomenon, I shall describe only the two most important experiments published in the Gazette.[b]

Experiment 1.[4] At 2:30 in the afternoon of August 20, that is, an hour and a half after I observed the phenomenon, I placed some ordinary polenta under a glass globe placed upside down on a plate containing

[4] The report of these experiments was published in the Gazzetta privilegiata, anno 1819, no. 190, martedi, 24 agosto.

water in such a way, however, that the polenta was more than an inch away from the water. At 11:00 in the morning of the following day, there appeared on the surface of the polenta some red spots, which multiplied so rapidly that the whole surface was colored by the evening of the same day—less than forty-eight hours from the time the experiment was begun.

I saw that as the reddening was taking place, it was well to remove the water and to renew the air within the globe. The temperature of the air was then about 21° Reaumur.

Experiment 2. I suspended some polenta in a place where the atmosphere was damp and continually changing, and where, in addition, foul exhalations were being emitted. Observing that the polenta was becoming dry, I moistened it with water a few hours after I began the experiment. In this case, the phenomenon made its appearance sooner than in the preceding experiment, and the red color was of the brightest hue imaginable.

These two experiments prove, as you will see, that this reddish matter of the polenta is produced in a very damp and warm atmosphere. If in addition to this, there are putrid exhalations in the air, then it is still more conducive to the production of the phenomenon.

These were my first deductions; but thinking since of a circumstance, to which Professor Innocente turned his attention, I suspected that the fact that I had already had some colored polenta in my hands and had kept some in my own house might have contributed to the formation of the red color on the polenta used in the experiments. In fact, if the colored matter that was produced had been a moldiness, we know with what fecundity these very minute plants are endowed, and how fine their seeds are, so that they could easily be borne anywhere.

This, then, being possible, I could no longer place any confidence in my experiments, and I became uncertain as to the production of the phenomenon by a warm, damp and foul atmosphere. The greatest obstacle presented by this new difficulty was that I could not clarify the matter by myself. But since necessity is the mother of invention, I wrote to Agostino Mano-

cchi of Mestre, a young man of distinguished talents, requesting him to repeat my experiments. He kindly consented to do so, and obtained very favorable results, whereby all doubts were then dissipated. Nevertheless, although the whole matter was made clear enough, I wished to repeat the experiments myself while I was in Padua in 1820. Again this time the phenomenon appeared as in the preceding year, although I had not had any colored polenta in my hands before the experiment. I did the same at Vicenza, and in various other places in the territory, and since then I have repeated the experiments at Venice. In all these cases, I obtained like results with a combination of the same circumstances. I must however, confess that in repeating Experiment I in 1820, 1821 and 1823, I did not get so much red matter on the surface of the polenta as I did in 1819. But this richness of color and of matter resulted when the experiments were performed in places near rice fields. Indeed, I observed that the coloring appeared sooner on the days when water was drawn from the rice fields, which fact strengthened my opinion of the influence of foul exhalations in the production of the phenomenon.

In this connection let me point out that Melo in his published articles makes mention only of humidity, omitting to say anything about foul air. He neglected to copy this latter truth (the influence of foul air) from me, but he was kind enough to indicate one of my mistakes,[5] expatiating at length on it instead of dismissing it with the severity which it deserved. And strictly speaking, I do not consider it to have been an error, having expressed myself thus: "It is a constant characteristic of polenta made with meal made from *Zea mays*, or Indian corn, to produce the coloring matter on its surface, whenever it is placed in a damp atmosphere, or subjected to foul exhaustions, perhaps because under such conditions, the polenta gives rise to spontaneous putrefactions." I would ask you to pay particular attention to the word "perhaps," and to read what I have written at the end of my article, and you will find that I said: "the nature of the red matter . . . . . is a subject which requires further investigation."

[5] See Da Rio, op. cit., t.c., pag. 334.

You see, then, that I was in doubt, and never asserted that the red coloring was a product of fermentation, since in only forty-eight hours I could not make enough observations to decide the nature of this substance. Melo, however, not paying careful attention to the word "perhaps" has asserted that this reddish substance was a product of fermentation. He confesses that he is not a chemist,[6] and we shall not argue with him on this point; but he asserts that he has read chemical books and therefore considers himself in a position to maintain his argument with assurance. He has fallen into this error just because he drew a positive conclusion from my doubt, instead of observing closely the circumstances attendant on such a phenomenon.

He seems to base his deductions upon the results of observations with the microscope, but this method of inspection should be only accessory, when more exact methods are available which allow us to distinguish organic beings from ordinary matter. I shall now inform you concerning Melo's experiments to determine the nature of the red substance.

Having asserted that the phenomenon is a product of fermentation, in order to remove all question that this was the case and that it was not caused either by a very minute plant, or an animal of the lowest classes, he dissolved the polenta in water and observed the colored substance with a microscope, but never did it appear *filamentous* or *oscillating* and therefore he felt his opinion was corroborated. I do not say that he was mistaken because of this, but I do think that having a better method for elucidating the phenomenon, this ought to be preferred, since our senses may deceive us. To settle this question, I therefore chose another method, taking as my guide the original experiments of that very brilliant scholar, Spallanzani.[7]

I had observed that by placing a little piece of colored polenta in contact with ordinary polenta, even though freshly prepared, in a very short time the surface of the polenta turned red. Again, I found that by bringing freshly made, warm, polenta

[6] See Da Rio, op. cit., t.c., pag. 336.

[7] See Spallanzani Opuscoli di fisica animale e vegetabile.

near the colored polenta, the red spots appeared, although there had been no actual contact. To obtain the coloring in this last case, I placed the red piece of polenta so that it would be in the physical center of a circle a little more than 2 inches in diameter, whose perimeter was formed by freshly prepared polenta. The central piece, then, was about an inch away from the uncolored polenta; but even in this case the uncolored polenta became red, and in a shorter time than in the preceding experiments.

I wanted next to see if the influence of the red polenta on the other polenta would continue to be manifest even if the distance were still greater, and if its effects were exercised in all directions.

I took, therefore, a glass globe whose diameter was 16 inches, and which had four apertures an inch wide, arranged in the form of a cross; that is, corresponding to the extremities of two axes, crossing each other at right angles. In this sphere or globe I suspended a little piece of colored polenta so that it would be in the physical center of the globe, and in each of the four apertures I placed a bit of freshly prepared polenta, while these pieces were still warm. In the course of a few hours, the red color showed itself to an equal degree in all directions, since all four pieces were colored in a like manner.

It seemed to me that this potent influence of colored polenta could not be ascribed to fermentation, since I cannot understand how polenta in three or four hours gives rise to a putrefaction capable of producing the phenomenon. Let it be said, moreover, that polenta so colored does not mislead us, by showing only the *mucilaginous substance tinted with red*, as it appeared to Melo, since there are visible also many clusters of very small hemispheres so that the surface looked like a substance sown with very minute pustules. Such experiments and observations led me to suspect from the beginning that the phenomenon might be produced by an organic being, rather than by fermenting matter.

If, therefore, the reddish substance were a living organism, it must be either a little animal of the class of the infusoria or a

very minute plant. Following the experiments of Spallanzani, if the colored matter resulted from an infusorian, I could kill it by exhalations of some volatile substances. If, therefore, before making the experiments, I subjected the red polenta to such exhalations, it could no longer exercise any coloring influence on fresh polenta; since, once the animals were killed by these deadly fumes, they could no longer generate those little eggs which, carried by the air, gave rise to a numerous progeny on the polenta on which they alighted.

That great naturalist, Spallanzani, found camphor vapors were the most deadly to animals of the class of infusorians.[8] Therefore, before doing anything else, I subjected the piece of colored polenta to a very strong camphor vapor and then repeated the experiment with this bit of polenta; but in spite of this the red coloring appeared as heretofore. I obtained like results even after subjecting the colored polenta to the strongest odors of turpentine and of tobacco; and only after prolonged subjection to sulphur fumes was the power to produce the phenomenon taken from the colored bit of polenta. Still I could not draw a sure deduction from this, since the strong acid produced in this case might well injure the germs of microscopic plants, in case these latter were the cause of the phenomenon. Indeed I must say that this was my principal doubt, and so I continued to follow the path of Spallanzani, who determined the degree of temperature at which the germinating faculty of certain seeds would cease.[9] If I, then, could deprive this colored polenta of its germinating faculty with a certain degree of heat (but never so great as to burn the polenta), then I would have valid reasons for concluding that the phenomenon was the product of a vegetable being.

I took, therefore, a little glass globe, and suspended my bit of colored polenta in its center; then I closed it with a cork through which I passed a small thermometer, the bulb of which was very near the colored polenta. After having sealed the

[8] See Spallanzani Opuscoli di fisica animale e vegetabile, tom. I, cap. VII, pag. 101.

[9] See Op. cit., cap. IV, pag. 45, 46, 47, ecc.

neck of the globe, I raised the temperature up to 80° Reaumur and I left it at this temperature for ten minutes. Then I took this bit of colored polenta and repeated the ordinary experiments; but the red color appeared just as soon and just as bright as before.

Seeing then that if this phenomenon was the result of a very minute plant, even 80° were not sufficient to deprive its seeds of vitality, thus I wanted to subject them to still greater temperatures to see if they would be made incapable of reproducing their kind. I buried the globe in sand, and at first raised the temperature to 100°, but finding no sensible difference in the results, I made another attempt and raised the temperature to 120°, and left the colored polenta at such a heat for only five minutes. Although in this last experiment I reduced the time to a half of that allowed in other experiments, yet it was sufficient, and perhaps too much so, to kill entirely the reproductive germs, and thus to prevent the red polenta from exercising any influence at all on the uncolored polenta although they were placed in actual contact. I repeated this experiment several times, always with the same results. Therefore this reddish substance obeys those laws to which Spallanzani found vegetable seeds subject. Reason, based on analogy, thus permits us to infer that this colored matter which grows on polenta is indeed among the lowest types, but is nevertheless a vegetable being.

I ought not to omit to say, however, that Spallanzani kept the spores of ordinary molds first at boiling temperatures and then at that of red-hot coals. In spite of such an intense heat, he affirms that the spores retained their germinating faculty.[10] In these experiments, however, he does not indicate the exact temperature of the coals, so that we do not know whether the degree of heat that killed the germinating faculty in this case was greater or less than that used by Spallanzani. It is certain that if the spores of these molds were exposed to red-hot coals, the decomposing power of the heat would have exercised its influence over them, and the entire mass of spores would have

[10] See Spallanzani, op. cit., tom. II, pag. 271.

been burned. Let it be added, moreover, that it is not easy to observe any difference in the growth of molds on sown substances from that on a body not sown with spores. This difficulty, in a large measure overcome by the singular skill of the great naturalist, has nothing to do with the case of my plant which under like circumstances grows on a substance on which seeds have alighted but not on one that is unseeded. I leave it to others to judge whether the path followed for this investigation into the nature of the colored matter is better or more certain than that followed by Melo. For my part I am not at all convinced by Melo's experiments and still less by the reasoning he sets up before ascertaining the true nature of the plant under discussion. These are his words:[11] "I am strengthened in this opinion by the fact, that if the red color of the polenta depended on some little plant this could be none other than a *Byssus*[4] or else a *Mucor*," as if, I may add, the Supreme Being could not create others of these microscopic beings besides the *Byssus* and *Mucor*. I feel sure that no one can accept such strange reasoning in regard to natural things. But he later informs us that that which produces the phenomenon is neither a *Byssus* nor a *Mucor*, and depriving us of a suspicion that it may be *Aegerita crustaceae*, he concludes that it can be nothing but a product of fermentation, concerning which I have already exposed the error.

Having now removed all doubts as to the nature of the colored matter, we have now to determine to what class of plants it belongs. And having instituted a rigid examination for this purpose I am inclined to place it in the order of fungi, possessing, as this plant does, all the characteristics pertaining to this order. Wishing to classify it more in detail, I find that, according to Bulliard's method, it belongs to the third group, in which, however, there is no type to which it can be referred. If I follow Persoon's system, then my plant belongs to the second class and to the fifth order; but neither here is there any type under which it can be classified, and I am therefore constrained to create a new genus.

[11] See Da Rio, op cit., t.c., pag. 340.

I thought it well to distinguish this new genus by the name Serratia to recall better[12] thereby to the memory of Italians the name of a celebrated physicist, whose memory is neglected so that we attribute to the foreigner that which exclusively belongs to us. Serafino Serrati was the first who plied a steam boat on the Arno,[13] and so, whatever be the merits of the claims of those beyond the sea, that of the invention certainly cannot be accorded to them. I therefore, have chosen to call the new plant by the name of Serratia to honor in this way the memory of this great physicist. The following are the generic and specific characteristics of the plant.

SERRATIA[a]

Funguli acaules, semisphaerici, capsulis confertis. S. Marcescens. Vesicula tenuissima latice primo roseo, dehinc rubro repleta.

I have distinguished my *Serratia* by the name *marcescens*, since as it reaches maturity (which is effected in a few hours) it decays immediately, dissolving into a fluid and viscous matter which has a mucilaginous appearance. This fact led Melo into error, as on seeing this substance, he without examining it, considered it a gum or mucilage, and so thought the phenomenon depended on the coloring only. He surely would not have been drawn into such a mistake, had he studied closely this tiny new plant in all the stages of its life, all of which stages are passed through in a few hours. If he had studied it in this way, he would have seen it in its early stages of development colored light pink, which changes into a dark purplish red,[14] a sign of the end of the plant's life.

[12] I have used the word *better* because the eminent Professor Pozzi has just taken occasion to recall Serrati with pride, and makes mention of him still more recently in his Dizionario di fisica e chimica, tom, II, pag. 308.

[13] See Lettera di fisica sperimentale, edizione di Firenze, anno 1787.

[14] Perhaps its beginning precedes this appearance of the aforementioned color a little, and I could mention some facts to support this belief, but since positive observations can only be made at the time when the color begins to appear, so I begin from this point, leaving out of account those first signs of life which appear before it becomes red.

If we examine it carefully in those brief moments of life assigned to it, we see nothing but little red spots formed by an aggregation of very minute, stemless fungi, covered with a very thin and clear skin, which under a microscope show some spots which appear of a duller hue scattered here and there. Seeing this, I suspected that the reproductive germs were perhaps contained in these spots. If we wish to use the reasoning based on these experiments, we see the doubt has been so clarified that to persist in suspicion would smack of over subtility. In fact, if the little seeds, or to use a modern botanical term, "spores," whereby the *Serratia* is reproduced, did not exist in individual cells in the skin, how could they be disseminated in the air and so alight on polenta placed at a distance? If, on the other hand, these seeds were in the interior, they would be held in the viscous fluid which is there contained, and could no longer fly through the air, and reach a distant substance on which to reproduce themselves. If this were the case, reproduction could be possible only by means of actual contact which fact has been disproven by experiments.

Let us suppose, then, that the more highly colored spots of the surface layer be so many tiny cells, which burst on reaching maturity. In such a case a mass of very minute seeds perhaps invisible even under a microscope, will be set free, and falling on those substances which possess the necessary characteristics for their development, will give rise in a short time to our little plant.

Even if the seeds are freed by the bursting of the little vesicles still not a few are retained and are absorbed in the rapid dissolution of the little plant, while some remain in the viscous fluid that is formed. Wherefore if the surface of a piece of paper be brought in contact with this fluid, the paper acquires the power to produce very rapidly our colored substance on polenta, in the same way as did the bit of colored polenta, except that the paper is effective only if it is in actual contact with the polenta. This, then, serves as a proof of the existence of cells in the surface layer of the *Serratia*, and also of their function. In fact, if there are on the polenta, already colored

and moistened before the experiment, some little plants in vegetation (a thing which we believe to be the case) these must hurl their seeds and propagate the species on bodies even if they be at a distance. This cannot occur in the case of ordinary paper that has been soaked, since it contains seeds immersed in the dried fluid, and these cannot become detached from the paper, and so contact alone can bring about reproduction.

Perhaps there are some who will not understand why the development of the phenomenon takes place sooner when we use a colored piece of polenta instead of paper soaked in the fluid. And in fact, Spallanzani,[15] sowing ordinary molds did not discover this precocious sprouting, and observed only a very slight difference between the germination of molds on inoculated substances and that on uninoculated material. In our case, however, it is well to note that the *Serratia* seeds are not found in the air in such great abundance as for example, the *Mucor* spores. Therefore, to get the coloring on polenta we must wait for several hours; an interval, which, however, is not due to the development of the plant, but rather to the time required for the air to bring the seeds in contact with the substance to be colored,—a period of time which is no longer necessary if we have recourse to methods explained above.

A very convincing proof of this may be obtained by repeating the first experiment described. In this case the piece of polenta under the globe will very often become colored only on one side, even after an exposure of eighteen to twenty hours. On this side there appear some spots constituting a stain, which spreads in a short time without any relation to the original piece of colored polenta, without which the first colored spots would be impossible. Very often the reddish color begins at only one point and gradually extends itself; nor can we always see any other spot on this same piece of polenta. I do not know how Melo can explain this phenomenon satisfactorily, which also occurs frequently with fermentation; since there can be no doubt that the colored bit of polenta is in equal relations to the other pieces. Therefore, he will never be able to tell us

[15] See Spallanzani, op. cit., tom. II, pag. 267, 268, 269, 270.

why all parts should not show a like fermentation. I am very sure that he will find it very difficult to explain this observation unless he abandons his first idea in regard to the matter. Indeed, if he agrees, as now he can no longer deny, that the colored matter is a plant, then he can tell us that only that portion on which the seeds fell became colored. And as the first plants to germinate reached maturity, they reproduced themselves and multiplied excessively, until with the rise of other molds, giants compared with these plants, these latter were suffocated and killed.

Spallazani thinks the germinative faculty of the seeds lasts many years.[16] In this regard, I can state only that the *Serratia* seeds as preserved in the paper, germinate after three years. I repeated these experiments every year, taking for the purpose a piece of polenta, still a little warm, which I placed under a glass globe on paper soaked in the colored substance. Each year I saw the *Serratia* develop as in 1819.

The power to preserve the seeds possessed by the impregnated paper is also possessed by wood, and perhaps by other substance soaked in the fluid. This observation explains how in the home of Pittarello of Legnaro and other farmers the polenta became colored in the period of a few hours. Indeed, in addition to supplying the conditions necessary for the development of the plant, these farmers, without realizing it, were sowing it by placing the polenta on the same board and in the same cupboard. In such a case the wood must receive some of the seeds, whereby the phenomenon was reproduced on the polenta that came in contact with it.

After having entertained you at such length on all that which concerns the life and history of this organism, I think it will please you to know something about the properties of the pigment which constitutes the greater part of the fluid of the *Serratia*.

The pigment is insoluble in water, and is dissolved readily in alcohol, even at ordinary temperatures. The solubility of the colored pigment in alcohol is another argument against

[16] See Spallanzani, op. cit., tom. II, pag. 272 e 273.

Melo's opinion, that the colored matter is mucilaginous. Thus we see that the fallacy of his reasoning becomes patent whether we have recourse to physics or chemistry. But to speak no longer of him, already sufficiently confuted, I wish to say that, having obtained the pigment by the method described, I was able to dye some silk and wool with the aid of ordinary mordants, so that I obtained some red shades, beginning with a delicate, pale red to a dark, purplish red. Having seen that, I thought I could derive some practical benefit therefrom, but further tests dissipated my hopes; since I found that light had a very powerful effect in decolorizing the tint produced by the new substance. And although the colored objects were not exposed directly to the light, still in the course of seven or eight months they faded, and after a year and a half almost every trace of color was lost, there remaining in its stead only a light yellowish shade.

On thinking about this I thought that perhaps the substances used as mordants might have contributed to the fading, and that possibly light was not the only decolorizing agent. To clarify this doubt, I colored some silk and wool without the use of mordants, and found that the fading occurred much sooner, and that direct light is so effective as to cause the color to disappear in a few moments.

What seemed singular to me was that light does not affect the color when it is associated with the plant. This leads me to think that there is some other principle which shields it from the force of light.

This is all that I have to say to you about the phenomenon which gave rise to so many strange ideas and fears, and I close with expressions of highest esteem.[1]

TRANSLATOR'S NOTES

[1] This letter originally appeared under the following title a little more than one hundred years ago in a journal published at Venice, Italy: Lettera di Bartolomeo Bizio al chiarissimo canonico Angelo Bellani sopra il fenomeno della polenta porporina. Biblioteca Italiana o sia Giornale di Letteratura, Scienze, e Arti, Tomo 30, Anno 8, Aprile, Maggio, Giugno, 1823, 275–295.

[b] Five experiments are reported in the article to which Bizio refers. In these he developed the red coloring on polenta in foul and in moist air, and found that it did not develop well under dry conditions. The article appeared anonymously but is later claimed by Bizio here and elsewhere.

[c] 212°F. or 100°C.

[d] *Byssus* a generic term of this period meaning a "thread" applied to various *Fungi imperfecti*. *Mucor*, the black molds, a generic term, used in a somewhat looser way than it is today.

[e] *Serratia*. Small, stemless fungi; hemispherical capsules occuring in clusters. *S. marcescens*. A very thin vesicle filled at first with a pink, then with a red fluid.

[f] A two and a half page note by Bellani follows this letter. In this, he discusses the possibility that the reddening of a pool of stagnant water that he observed in the fall of 1819 was caused by the same organism. This pool called by the peasants, "the red sea" evidently contained certain red algae.

THE TYPE SPECIES OF THE GENUS SERRATIA, COMMONLY KNOWN AS BACILLUS PRODIGIOSUS[1]

R. S. BREED AND MARGARET E. BREED

New York Agricultural Experiment Station, Geneva, New York

Received for publication May 10, 1924

In the recent divisions that have been made of the poorly defined and unwieldy genera, *Bacterium* Ehrenberg and *Bacillus* Cohn, some confusion has arisen regarding the selection of the type species of the red chromogenic, non-spore forming rods, and also regarding the correct scientific name to use for this species. For this reason, the authors of this paper have recently undertaken the study of a series of cultures of organisms of this group, and a review of the literature pertaining to them. The present paper presents our conclusions regarding the selection and naming of the type species for the old, but only recently recognized genus *Serratia* Bizio (1823, p. 288).

In 1918, Buchanan (1918, p. 51) reached the conclusion that the proper generic term to use for these rods was *Serratia*, and that the type species of the genus was *S. marcescens* Bizio, the organism more commonly known to bacteriologists as *Bacillus prodigiosus* (Ehrenberg) Flügge. Even earlier than this, Vuillemin (1913, p. 518) had concluded that the validity of the genus should be recognized, and had pointed out that, by right of priority, it should be used in place of one of the more commonly used terms such as *Bacillus* or *Bacterium*. He therefore proposed to select *Serratia subtilis* (*Bacillus subtilis* Cohn) as type for the revived genus *Serratia*. As this suggestion contravenes well recognized rules of nomenclature, and is appropriate only

[1] Presented before the Society of American Bacteriologists, New Haven, Conn., December 27, 1923.

in case we wish to retain a very broad definition of the genus, it does not concern us further at this time.

The acceptance of Buchanan's suggestion causes the substitution of an unfamiliar name for a familiar name of a well-known and common organism so that the Committee on Classification of the Society of American Bacteriologists[2] in their final report (1920 p. 209) have suggested the use of *Erythrobacillus* Fortineau (1905) for the red chromogenic rods with *Erythrobacillus prodigiosus* (Ehrenberg) Committee as the type species. The Society also took action approving this use of *Erythrobacillus* at the Boston meeting in 1919. However, since this action was taken, various important considerations have been brought forward which change the situation. Enlows (1920, p. 41) for example, has shown that the species that must be regarded as the type for the genus *Erythrobacillus* is *E. pyosepticus*, the only species included in the genus by its author. As this species is a little known and possibly unidentifiable one, the acceptance of *Erythrobacillus* causes complications. Moreover, this suggestion, as is shown later, is not an entirely happy one as it disregards early Italian work which was of high order for the period in which it was done. For these reasons, the use of *Erythrobacillus* does not seem likely to win the general international support that all scientific names should have.

A detailed study of the early literature by the authors of the present paper has shown that Buchanan's suggestion is founded on a correct appreciation of the facts so that the recommendation was made that *Serratia* be used in the Manual prepared by Bergey (1923, p. 85).

The basis for this conclusion is found in study of the early work done by Bizio and Sette, a study carried out in the district of northern Italy where these men did their work, and in a partially completed study of a series of cultures of red chromogens

[2] The action of the Committee in adopting the name "*Erythrobacillus*" was taken in accord with the principle endorsed by the Society December 29, 1919, "That the date of publication of the third edition of Zopf's Spaltpilze (1885) be considered the beginning of bacterial nomenclature for the purpose of determining priority, with the exception of a definite list of genera conservanda."

which included one of so-called *Erythrobacillus* secured from the Pasteur Institute in Paris. The latter is probably a sub-culture of Fortineau's organism.

HISTORY OF THE EARLY WORK ON SERRATIA MARCESCENS

Although there is a long and romantic history connected with the appearance of "bleeding" bread, "blood drops" on sacred wafers, and the like that were probably caused by the organism here discussed,[3] it is not the purpose of the present paper to go back of the outbreak of so-called "bleeding" polenta (corn meal mush) that occurred among the peasantry of the little village of Legnaro near Padua, Italy, about 100 years ago. It was as a result of fine work by Bizio and Sette at this time that it was first recognized that this phenomenon was caused by a living organism (fungus) similar in many of its manifestations to the little plant (alga) causing the red snow of the alpine districts. Copies of the complete and truly scientific reports of this work have previously been inaccessible to modern students. These rare papers were fortunately found at the Biblioteca Marciana, Piazza San Marco, Venice, and phototostatic copies of them were secured through the courtesy of the Librarian, Dr. Pietro Zorzanello. The authors are also under obligation to Prof. C. P. Merlino of Hobart College, Geneva, N. Y., for aid in translating these old Italian works. He has also prepared a complete English translation of the article by Bizio (1823) which includes the description of the organism that appears to have priority. The translation will be found on the pages immediately preceding the present communication.

The story of this outbreak as told by various authors (Anonymous, (1819, 1819a), Melo (1819), Bizio (1823, 1827, 1844) and Sette (1824)) may be briefly given as follows: No little excitement was caused among the superstitious peasantry about Padua in July, 1819 by the appearance of "blood" on

[3] See Hefferan (1903) and Scheurlen (1896) for recent accounts of this history. Their accounts are largely taken from the less accessible but more complete story worked up by Ehrenberg (1849, 1849a, 1850, 1851, 1851a).

polenta stored in a cupboard belonging to one Antonio Pittarello. The disturbance became so great that the police authorities were forced to take cognizance of the matter and appointed a commission to investigate. This was largely composed of professors from the University of Padua. Others became interested and studied the matter independently. Among these was a young pharmacist, Bartolomeo Bizio, who was apparently the first to attempt a natural explanation of the phenomenon. This appeared as an anonymous publication (Anonymous, 1819a) in the Official Gazette at Venice, but is claimed by Bizio in his account published in 1823, and he repeats this claim in 1827 and 1844. Others like Dr. Vincenzo Sette, a physician and surgeon of Piove, who accompanied the official commission in their studies of the matter at Legnaro, came to a similar conclusion. On the other hand Dr. Pietro Melo, Director of the Botanical Garden at Saonara (or Savonara), claimed that the phenomenon was due to a spontaneous fermentation of the polenta which caused the corn meal to be transformed into a colored mucilage.

Melo's paper was the first to appear in print and it is this paper by Melo that Bizio had seen before his publication in 1823. In this he combats Melo's ideas vigorously. His discussion is a most interesting forerunner of the great controversy that was to take place about forty years later between Pasteur and Liebig over the nature of fermentation. Bizio shows himself an apt pupil of the great Spallanzani (Locy, 1910) whose work in combating erroneous ideas in regard to spontaneous generation laid the foundation for Pasteur's work in the same field. Bizio's work is also of great interest because it shows him using methods of cultivating chromogenic bacteria on a solid medium (corn meal mush) that antedate Schroeter's similar work by fifty years. In this paper, Bizio names the organism *Serratia marcescens*.

Sette's equally careful and scientific work was presented before the Atheneum at Treviso on the evening of April 28, 1820, but he was less fortunate in securing publication. Even the report of the Proceedings of the Society put out by the Secretary, F. Amalteo (1824) did not appear until 1824 although the date

1820 is sometimes incorrectly used because the meeting took place in that year. Sette's description of the fungus appears as a footnote on page 57 of this report. Sette's complete report (Sette, 1824) appeared as a pamphlet published in Venice and there is no way of telling whether it antedates the publication of the Proceedings by the Secretary of the Society. In either case, since both bear an 1824 date, it is clear that Bizio's description has priority.

Bizio rearranged the material in his 1823 paper with some additional comments on Sette's work in his two volume work entitled "Opuscoli Chimicofisici" published in 1827. In this, his Latin description of *Serratia marcescens* is reprinted on page 289 in exact duplication of the original in the 1823 paper. However, in his (Bizio, 1844) paper published in the Proceedings of the Academy of Science at Paris, in which he defends the priority of his work at Legnaro and points out the probable identity of the phenomenon causing bleeding polenta with that causing an outbreak of bleeding bread in a French garrison, two changes appear in the description that are evidently typographical errors. One is in the spelling of the specific name which appears as "marescens" and the other is in the substitution of the word "contortis" for "confertis." Hefferan (1903, p. 314) in copying this already incorrect copy has added another error in giving the second word as "caules" instead of "acaules."

Sette's Latin description as given below appears on page 51 of his pamphlet. This description appears in identically the same form in Amalteo's report except that the footnote references are lacking and the abbreviation *Car.* is given more completely as *Caract.*

Genus Zaogalactina (32). Car. Receptaculum nullum, substantia gelatinosa similaris, forma constanti, generatio obscura (34).

Species Zaogalactina Imetrofa. Car. Granuliformis, minima, gregaria, sessilis, intense punicea.

Observatio. Ad superficiem quorumdam alimentorum aestate anni 1819 in humilioribus Provinciea Patavinae regionibus. Copiosissime observata.

The figure (32) in the original is a misprint for (33). The figures refer to notes at the end of the paper in which the derivation of the name is given as meaning "living gelatin found on food."

A free translation of the Latin description would read:

> Genus Zaogalactina. Characteristics. No receptacle. A gelatin like substance of constant form, whose method of reproduction is obscure.
>
> Species Zaogalactina imetrofa. Characteristics. Round in form of small size, occurring in clusters. Sessile. Deep blood red.
>
> Occurrence. Observed on the surface of certain foods in the summer of 1819, in humid regions of the Province of Patavina. Occurs abundantly.

It is well to note the spelling of this binomial, as a peculiar fate has followed Sette's name, due to its having appeared in such an inaccessible publication. Montagne (1853, p. 529), Schroeter (1872, p. 110) and Trevisan (1879a, p. 62) give it as *Zoogalactina imetropha*, while De-Toni and Trevisan (1889, p. 976) use *Zaogalactina imetropha* and *Bacillus imetrophus*. Chester's incorrect spelling (1901, p. 258) *Zoogalactina immetropha* is copied by Hefferan (1903, p. 314) while Enlows (1920, p. 98) quotes the original spelling *Zaogalactina* as if it were incorrect and incorrectly gives *Zoagalactina*. Bergey (1923, p. 87) adds still another spelling in *B. impetrophus*. There seems to be no good reason for changing the spelling from the original though other spellings may be preferred by some.

Although Bizio's and Sette's descriptions were clearly drawn up independently, they are worded very much alike and are in the form used in systematic work of this period. The descriptions apply very accurately to the clustered pink and red colonies as they first appear on corn meal mush. Later the material becomes entirely overgrown if sufficiently moist and is transformed into a gelatinous mass. The young, round and hemispherical colonies were evidently regarded by both observers as stemless fungi, similar to mushrooms.

Conclusions similar to those reached by Buchanan (1918) and ourselves regarding the justice of Bizio's claim to priority have also been reached by Spica (1900) and confirmed by him as his present views in an interview with one of the authors of this paper at Padua in 1923. Professor Spica is the present Director of the Pharmaceutical Institute of the University of Padua, and his views have double value because he is acquainted with the rare publications discussing this outbreak at Legnaro. The historic institute of which he is the director may well have been the institution at which the young pharmacist Bizio received his training. It is situated on the Via Fallopio, a name that reminds us of the splendid scientific history of this famous university. It seems especially fitting that the credit for the discovery of the natural cause for the so-called miraculous appearance of "blood drops" on various foods should also go to men from this university, as the superstitions connected with this natural phenomenon have been responsible for many executions and murders of innocent persons in Italy as elsewhere in Europe.

Although these discoveries were reported a century ago, it is evident that superstitious and ignorant peasantry may still be excited by the growth of Bizio's organism on food. A colleague on the Experiment Station Staff who has lived many years in Italy states that the newspapers of Naples reported the disturbances that were excited in that city as recently as 1910 or 1911 when a "bleeding host" was found in one of the churches. This caused many ignorant people to seek the protection of the priests as did the similar outbreak in northern Italy.

Following the work done by Bizio and Sette, the next most important work was that by Ehrenberg. His interest in the matter began in 1848 when his attention was called to red spots on cooked potato in a home where there had been a fatal case of cholera. At first quite ignorant of the earlier Italian work, he later obtained a copy of Sette's pamphlet but remained throughout ignorant of Bizio's work and of his discussion of the outbreak in France four years earlier. With good reason he regarded the phenomenon that he had observed as identical with that seen by Sette. While Ehrenberg's observations and experiments

with the organism are not as complete as those of Bizio or Sette, he studied the history of similar outbreaks much more completely and also had the advantage of studying the material under the improved microscopic lenses of the period. From the microscopic examination, he discovered that the red material was made up of incredible numbers of tiny, isolated, oval animalcules which, because of their motility he regarded as animals. He even goes so far as to imagine that he saw a single polar flagellum in one of his dried preparations. This was perhaps half as long as the body. Reproduction is stated to be by longitudinal fission. Because of these conceptions, the majority of which are now known to be erroneous, Ehrenberg proposed to substitute a new name for the organism in place of the name given by Sette. Ehrenberg's description (1849a, p. 359) is as follows:

Monas prodigiosa (= *Mucor sanguineus* de-Col = *Zaogalactina imetrofa* Sette) corpusculis $\frac{1}{3000}$ – $\frac{1}{8000}$ lineae longis, subrotundis, singulis hyalinis, acervatatis sanguineis, proboscide corpore breviore. In cibis humidis interdum copiosae sanguineas maculas gelatinosas efficiunt.

In pollice cubico uno 46,656,000,000,000 ad 848,736,000,000,000 vivunt. Habitat in Syria et Europa.

A free translation of this would read:

Monas prodigiosa. Corpuscles from $\frac{1}{3000}$ to $\frac{1}{8000}$ lines in length, ovoid, when single, hyaline, when massed together, blood red, bearing a flagellum shorter than the body. Occasionally found on moist food as abundant blood red gelatinous spots. The number living in a cubic inch varies from 46,656,000,000,000 to 884,736,000,000,000. Habitat Syria and Europe.

It is interesting to note that $\frac{1}{8000}$ of a line is a little less than 0.5 μ while the larger dimension would be about 0.75 μ figures that are commonly given as the size of these bacteria. The number per cu. in. is undoubtedly based on computations made with a microscope. The habitat given is due to Ehrenberg's knowledge of the numerous occurrences of this organism in various parts of Europe and in the bread of Alexander the Great's troops in their siege of Tyre.

Ehrenberg's assignment of this organism to the animal kingdom was soon challenged by Montagne (1853). The latter's interest in the matter arose through the appearance of the organism in its characteristic way on some cooked chicken that had been stored over night in a cupboard in a warm kitchen. This happened on July 14, 1852, while the author was visiting a friend in the country near Rouen, France. Montagne, who had just read Ehrenberg's work and who was also familiar with Sette's work reported on the matter to the agricultural society of Paris soon afterward. In his report, he disputes Ehrenberg's claim that the organism is an animal, and assigns it to the algae under the genus *Palmella*. Specimens were displayed showing the organism growing on cooked rice and on chicken. The author stated that even under a magnification of 1200 diameters he was unable to find the flagellum mentioned by Ehrenberg and that he regarded such movement as he found as being caused by an exaggerated Brownian movement caused by the very small size of the organism. In the discussion of the paper, it was pointed out that various reproductive cells of the algae were known to be able to swim by means of flagella so that even though flagella were present, Ehrenberg was not for this reason alone justified in calling the organism an animal.

DISCUSSION OF NOMENCLATURE AS APPLIED TO SERRATIA MARCESCENS

Among the generic terms that have been applied to this organism, the following are properly applied to species of algae rather than of bacteria: *Protococcus* as used by Meneghini (1848), *Palmella* as used by Montagne (1853) and *Micraloa* as used by Zanardini (1863). Among other terms suggested there are several that are today applied to genera of bacteria, but in each case the present tendency is either to drop the term or to so limit the genus as to exclude Bizio's organism. This statement applies to *Bacteridium* of Schroeter (1872), *Micrococcus* as used by Cohn (1872), *Bacillus* as used by Flügge (1886) and *Bacterium* as used by Lehmann and Neumann (1896). *Coccobacterium* as used by

Schmidt and Weis (1901), *Liquidobacterium* as used by Orla-Jensen (1909) and *Erythrobacillus* as used by Fortineau (1904) and Winslow et al. (1920), like *Zaogalactina* of Sette are invalid synonyms of *Serratia* Bizio. *Monas* as used by Ehrenberg is now used only for a genus of Protozoa so that it is no longer available.

Among these names *Serratia* has clear priority and should be accepted unless general usage indicates that a return to this early name would be unacceptable to a great majority of bacteriologists. However, a glance at the list of synonyms on a following page will show at once that there is not and never has been any general international usage regarding the generic term applied to this species. From this standpoint there appears to be no good reason for disregarding the law of priority.

The situation in regard to the specific name *marcescens* is not so fortunate, as Ehrenberg's specific name *prodigiosa* has been much more generally used, though various Italian workers have followed either Bizio or Sette. In order to retain Ehrenberg's specific term some one of the genera suggested since his time must be chosen. When this is attempted it will be found that this causes complications in every case as has already been shown for *Erythrobacillus*. Because of these difficulties and because the specific name *prodigiosa* perpetuates the erroneous conclusions drawn by Ehrenberg in place of the splendid pioneer work done by Bizio, it seems far better to accept *Serratia marcescens*, retaining the term of miracle (prodigium) bacterium for use as a trivial or common name.

Synonymy

The preliminary study of the series of cultures has already shown that this somewhat variable organism has been renamed several times since 1848 by workers who thought they had something new. Proper determination of this fact is always difficult and can only be made after the study of the cultures is completed so that a complete list of synonyms cannot be prepared at this time. It is, however, evident that all of the following combinations are exact synonyms of *Serratia marcescens* Bizio (1823, p. 288).

Synonyms—*Zoogalactina imetrofa* Sette (1824, p. 51); *Protococcus imetrophus* Meneghini[4] (1838); *Monas prodigiosa* Ehrenberg (1849, p. 359); *Palmella prodigiosa* Montagne (1853, p. 527); *Micraloa prodigiosa* Zanardini[4] (1863); *Bacteridium prodigiosum* Schroeter (1872, p. 110); *Micrococcus prodigiosus* Cohn, (1872, p. 153); *Micrococcus metrophus* Trlevisan, (1879, p. 19); *Bacillus prodigiosus* Flügge (1886, p. 284); *Bacilus imetrophus* Trevisan (1887, p. 799); *Bacillus marcescens* De-Toni and Trevisan, (1889, p. 976); *Bacterium prodigiosum* Lehmann and Neumann (1896, p. 259); *Liquidobacterium prodigiosum* Orla-Jensen (1909, p. 338); *Erythrobacillus prodigiosus* Winslow et al. (1920, p. 209).[5]

REFERENCES

AMALTEO, F. 1824 Relazione dell'altro parte dei lavori fatti durante dell'anno accademico 1819–1820 (Report of the other part of the work done during the course of the academic year 1819–1820). Memorie scientifiche e letterarie dell' Ateneo di Treviso, **3**, 56–58.

ANONYMOUS 1819 Nuovo Osservatore Veneziano, August 24, 1819, no. 101. 4.

ANONYMOUS 1819a Trovata della precisa cagione, che produce il fenomeno del superficiale coloramento in rosso della polenta (The explanation of the true reason for the red coloring of the surface of corn meal mush). Gazzeta privilegiata di Venezia, August 24, 1819, no. 190, 3–4.

BERGEY, D. H. 1923 Manual of Determinative Bacteriology. Williams and Wilkins, Baltimore, xii + 442 pp.

BIZIO, BARTOLOMEO 1823 Lettera di Bartolomeo Bizio al chiarissimo canonico Angelo Bellani sopra il fenomeno della polenta porporina. Biblioteca Italiano o sia Giornale di Letteratura, Scienze e Arti. Appendice Tomo 30, Anno 8, Aprile, Maggio, e Giugno, 275–295. (See English translation by C. P. Merlino).

BIZIO, BARTOLOMEO 1827 Del fenomemo della polenta porporina (The phenomenon of red corn meal mush). Opuscoli Chimicofisici, Tomo 1, Parte 2, Richerche e spiegazioni di alcuni fenomeni, Articolo 1, 261–298, Giuseppe Antonelli, Venezia.

[4] Original not found, Quoted from Trevisan (1887, p. 799).

[5] The term *Mucor sanguineus* De Col also sometimes appears in lists of synonyms. This term is ascribed by Sette (1824, p. 19) to De Col, a chemist from the University of Padua, who studied the outbreak at Legnaro and who became interested in the possible commercial value of the pigment extracted from these organisms. However, De Col does not appear to have ever published this name. At least no trace of the publication could be found either in the library at Venice or at the University of Padua. As it does not appear to have been effectively published, it is a hyponym, not a synonym.

Coccobacterium is given by Schmidt and Weis (1901, p. 9;1902, p. 10) as a generic term and they list *Bacillus prodigiosus* as the typical organism of this genus. However, the binomial *Bacillus prodigiosus* is used throughout the book.

Bizio, Bartolomeo 1844 Sur une altération du pain causée par le développement d'un cryptogame: observation faite, il y a très-longtemps, d'un phénomène qui rentre dans la classe de ceux sur lesquels Möser a appelé l'attention. Compt. rend. Acad. Sci. Paris, **18**, 951–954.

Buchanan, R. E. 1918 Studies in the nomenclature and classification of the bacteria. V. Subgroups and genera of the Bacteriaceae. Jour. Bact., **3**, 27–61.

Cohn, Ferd. 1872 Untersuchungen über Bacterien. Beiträge z. Biol. d. Pflanzen, Bd. 1, Heft 2, 127–224.

De-Toni, J. B., and Trevisan, V. 1889 Schizomycetaceae, Naeg. P. A. Saccardo's Sylloge fungorum, **8**, 923–1087.

Ehrenberg, Chr. G. 1849 (Observations on the so-called bleeding bread caused by *Monas prodigiosa*.) Bericht ü. d. z. Bekanntmachung geeigneten Verhandlungen d. Kgl. preuss. Acad. d. Wissenschaften, October 26, 1848, 349–353.

Ehrenberg, Chr. G. 1849a Fortsetzung der Beobactung des sogenannten Blutes im Brode als *Monas prodigiosa*. (An appendix to the previous article prepared after the first paper was read before the Academy.) Ibid. 354–362.

Ehrenberg, Chr. G. 1850 Fernere Mittheilungen über *Monas prodigiosa* oder die Purpurmonade. Ibid, March 15, 1849, 101–116.

Ehrenberg, Chr. G. 1851 Höchst wahrscheinlicher Grund d. Verbotes d. Bohnengenusses bei. d. Pythagoräern. Ibid, January 17, 1850, 5–9.

Ehrenberg, Chr. G. 1851a Eine Centurie historischer Nachträge zu den blutfarbigen Meteoren und sogenannten Prodigien. Ibid, June 27, 1850, 215–246.

Enlows, Ella M. A. 1920 The generic names of bacteria. Hyg. Lab., U. S. Public Health Service, Bull. 121, 111 pp., Washington.

Fortineau, Louis 1904 L'Erythrobacillus pyosepticus et les bactéries rouges. Thèse Fac. méd. Paris, 164 pp. Condensed report in Comp. rend. Soc. Biol. Paris, 1905, **58**, 104–106. Review in Bull. Inst. Pasteur, 1905, **3**, 13–14.

Flügge, C. 1886 Die Microorganismen. F. C. W. Vogel, Leipsig, xviii + 162 pp.

Hefferan, Mary 1903 A comparative and experimental study of bacilli producing red pigment. Centralbl. f. Bakt., Abt.II, **11**, 311–317, 397–404, 456–475, 520–540.

Lehmann, K. P., and Neumann, R. 1896 Atlas und Grundriss der Bakteriologie. J. F. Lehmann, München, vii + 448 pp.

Locy, W. A. 1910 Biology and its makers. Holt and Co., New York, xxvi + 469 pp.

Melo, Pietro 1819 Memoria sulla polenta rossa. Giornale dell'italiana letteratura del Da Rio. 49, 333–341, Padova (Fascicolo 2, Bimestre di nov. e dic., 1818).

Meneghini, 1838 See Trevisan, 1887, p. 799.

Montagne, C. 1853 Coloration rouge des substances alimentaires par la présence du *Monas* (*Palmella*, Mihi) *prodigiosa*, Ehrenb. (Séance du 28 juillet 1852). Bul. Soc. nat. et cent. d. agric. Paris, 2 Ser., **7**, 527–530.

ORLA-JENSEN, S. 1909 Die Hauptlinien des natürlichen Bakteriensystem. Centralbl. f. Bakt., Abt. II, **22,** 305–346.

SCHEURLEN, 1896 Geschichtliche und experimentale Studien über den Prodigiosus. Arch. f. Hyg., **26,** 1–31.

SCHMIDT, JOHS., AND WEIS, FR. 1901 Bakterierne. Copenhagen, v + 351 pp. Translated from Danish into German by Morten Porsild. Die Bakterien. G. Fischer, Jena, 1902, vii + 416 pp.

SCHROETER, J. 1872 Ueber einige durch Bacterien gebildete Pigmente. Beiträge z. Biol. d. Pflanzen, Bd. 1, Heft 2, 109–126.

SETTE, VINCENZO 1824 Memoria storico-naturale sull'arrossimento straordinario di alcune sostanze alimentose osservato nella provincia di Padova l'anno MDCCCXIX (A natural history memoir which discusses an extraordinary reddening of certain food substances. Observed in the Province of Padua in 1819). Letta all' Ateneo di Treviso nella sera 28 Aprile 1820. (Read at the Atheneum of Treviso on the evening of April 28, 1820.) Alvisopoli, Venezia, 63 pp.

SPICA, P. 1900 Sulla materia colorante prodotta dal *Micrococcus prodigiosus*. Rivendicazione di priorita par Bartelomeo Bizio (The pigment produced by *Micrococcus prodigiosus*, with a defense of the claims for priority made by Bartelomeo Bizio). Atti del Reale Instituto Veneto di Scienze, Lettere ed Arti, Anno 1899–1900, **59,** Parte 2, 1025–1031.

TREVISAN, V. 1879 Prime linee d'introduzione allo studio dei Batterj italiani. Rendiconti R. Istituto Lomb. di Scienze e Lettere, Ser. 2, **12,** 13–21, 133–151.

TREVISAN, V. 1879a Sulla causa dell'asfissia e dell'agglutinazione dei globuli del sangue nella diferite. Ibid, Ser. 2, **12,** 54–62.

TREVISAN, V. 1887 Se sia vero che il Bacillo imetrofo (*Micrococcus prodigiosus* di antichi autori) preceda sempre come agente specifico predisponente il calcino del baco da seta. Ibid, Ser. 2, **20,** Fasc. 20, 797–804.

VUILLEMIN, PAUL 1913 Genera Schizomycetum. Annales Mycologici (Sydow), **11,** 512–527.

WINSLOW, C.-E. A. ET AL. 1920 The families and genera of the bacteria. Jour. Bact., **5,** 512–527.

ZANARDINI, 1863 See Trevisan, 1887, p. 799.

THE EFFECTS OF SOME ELECTROLYTES ON THE BUFFERING CAPACITY OF BACTERIUM COLI

H. J. SHAUGHNESSY AND I. S. FALK

Department of Public Health, Yale School of Medicine, New Haven, Connecticut

Received for publication June 1, 1924

I. INTRODUCTION

Studies which have been reported from this laboratory on the relation of hydrogen ion concentration to viability of *Bact. coli* (Winslow and Falk, 1923) have suggested the great importance of certain amphoteric reactions of the bacterial cell. The experiments recorded here were undertaken with the view of studying, from another angle, certain anomalous results of the studies mentioned above. These anomalies are fully explained in the paper cited above but may perhaps be briefly mentioned here. It was found that certain sodium chloride solutions which were initially alkaline and in which the hydrogen ion concentration was not regulated showed toxicities to *Bact. coli* comparable with slightly acid solutions. On the other hand calcium chloride solutions starting at the same unregulated alkalinity showed toxicities which were comparable with those in alkaline rather than those in acidic solution. As a matter of fact the percentage viability in calcium chloride solutions at unknown pH (unknown because the experiments were first conducted without measuring or controlling the hydrogen ion concentration) was not comparable with the viability at any one pH value in similar solutions in which pH was held sensibly constant by repeated adjustments. It appeared that the explanation for these anomalous toxicities was found to lie in the fact that in sodium chloride solutions, as in water, there was a general shifting of the pH of the suspension *toward* neutrality (the most favorable zone for the viability of *Bacterium coli*); and that in these solutions of calcium

chloride, on the contrary, this shifting of pH was very much retarded. It was found, however, that the shift of pH in water and in sodium chloride solution was insufficient to bring the suspension *into* the zone of favorable viability and hence could not account completely for the observed results. The authors then conceived the idea that there might be a minute zone of solution immediately surrounding the bacterial cell which was buffered to a favorable pH by cell metabolites before the bulk of the menstruum had been similarly modified. In a test of this idea made by shaking part of each of a series of suspensions and leaving the remainder of each suspension unshaken, it was found that the pH of a shaken solution was shifted toward pH 7 more rapidly than the pH of its unshaken aliquot, due, according to this theory, to the dissemination of the buffering substances first contained in these minute zones contiguous to the cells throughout the solution in general nearly as rapidly as they were excreted. Experimental findings had already indicated that $CaCl_2$ reduced the rate of this type of buffering more than NaCl. Hence it appeared that the anomalous toxicity of alkaline $CaCl_2$ solutions could be accounted for on these grounds. It therefore seemed to the authors that it was of some importance to find a measure of the buffering capacity of *Bacterium coli* and the effect of hydrogen ion concentration and of calcium and sodium chlorides upon this capacity. The titration curve method was selected and utilized in the studies reported in this paper.

II. METHOD OF MAKING TITRATION CURVES

Titration curves have been used for the measurement of buffer in chemical systems (Clark, 1920, 1922; Cohn, Gross and Johnson 1919-1920, etc.) and in bacteriological media (Brown, 1921), but we have found no reference to their use in regard to the buffer of the bacterial cells themselves. Hence a description of the method of construction may be pertinent, particularly because it became necessary to make some modifications in the technique to meet the requirements of this study. Briefly, a titration curve is a graph made by plotting pH on one ordinate against

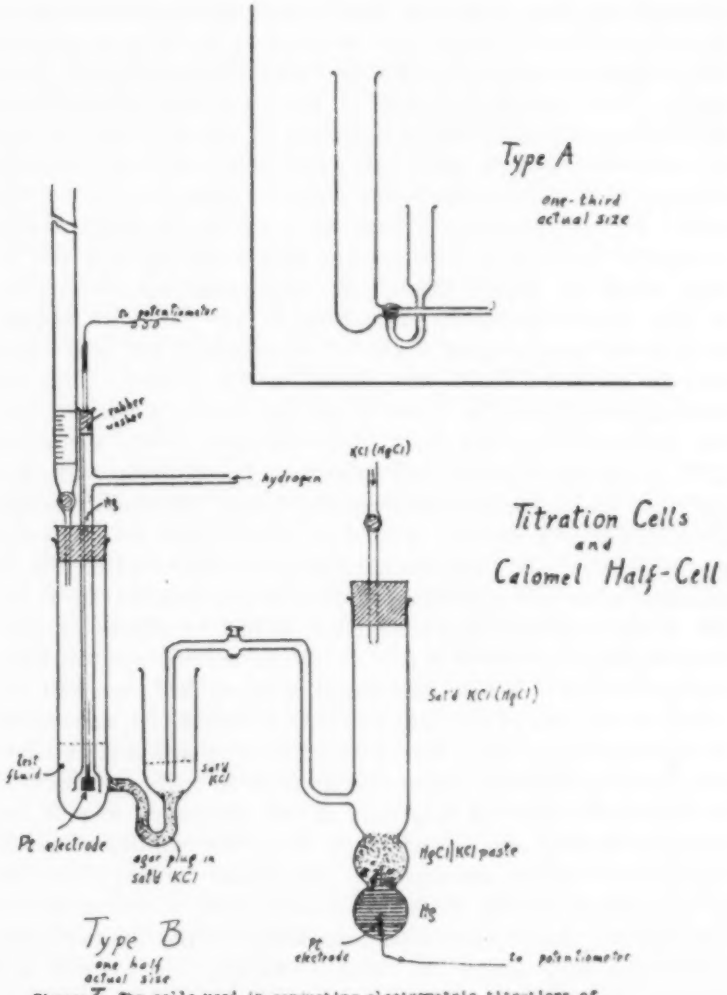

Figure 1. The cells used in conducting electrometric titrations of bacterial suspensions.

Fig. 1

measured amounts of acid or alkali added on the other ordinate. In our experiments the pH was determined by using an electrometric method briefly described by Falk and Shaughnessy (1922-1923). The essential portion of the apparatus differed from the ordinary titration cells as described by other workers in that we were compelled to use a cell which permitted but a minimal diffusion of KCl from the liquid junction solution into the test fluid. In our first experiments we used the chamber shown as type A in figure 1, in which the KCl comes up from the U-tube below to diffuse around the ungreased middle portion of the closed stopcock. The type B was later developed. In this cell an agar plug made by dissolving 1 per cent shred agar in saturated KCl was placed in the U-tube. This arrangement permits the flow of current to the potentiometer and galvanometer, but very little diffusion of the saturated KCl. The test solution was stirred by the escape of hydrogen gas from the modified Hildebrand hydrogen electrode through the solution. Additions of acid or alkali were made to the suspensions in the titration chambers in cubic centimeters or fractions of a cubic centimeter of 1/500 normality until the pH of the solution approached an acidity or alkalinity that required large additions of acid or alkali for further appreciable changes in pH. When that point was reached, we had recourse to the use of N/5 alkali or acid to bring the suspension to higher or lower pH. The quantity of suspension introduced into each chamber was either exactly 20 or 40 cubic centimeters. In the construction of the curve it was necessary to plot the grams of acid or alkali (computed from the cumulative cubic centimeters added) as negative logarithms to keep the size of the graph within bounds. Hence both coordinates are logarithmic. In all cases when calculating acidity the necessary corrections were made for volume changes due to acid and alkali additions. In this respect the data therefore represent titrations at constant volume.

III. MEASURING BUFFER FROM THE CURVES

It has been possible in the past to measure roughly the buffer of an ampholyte by finding the change in the slope of the titration curve (Clark 1919, 1920; Cohn, Wolbach, Henderson and Cathcart, 1918-1919), i.e., the further the slope of the line deviates towards the vertical the greater the buffer when pH units are represented as abscissae. It has been possible for us, however, to develop a method for quantitating buffer by the following means: first, a titration curve of the water or salt solution without bacteria was plotted and the grams of acid, let us say, to move the solution from pH 4 to 3 was read off from the curve; then a titration curve of water or of salt solution of this same strength, as the case might be, containing about 4000 million *Bact. coli* per cubic centimeter was inspected and the grams of acid necessary to move this suspension through the same pH zone, i.e., from 4 to 3, was read off. The ratio of the latter weight to the former gives what we are calling the buffer ratio. A similar ratio has been plotted for each pH zone except where the solution and suspension started at different zones or where part of the shift of one zone was made by alkali and part by acid. If there were no buffer, this ratio would equal unity; if a measurable buffering capacity were present, the ratio would be greater than one, in a degree depending upon the magnitude of this capacity. It is possible also to get a ratio of less than unity, implying perhaps that the cell is liberating its content of acid or alkali or that amphoteric constituents of the cell are dissociating, under the influence of the added acid or alkali, to increase the hydrogen or the hydroxyl ion concentration. However, in this measure of buffer we have a method which, while not postulating any particular theory to account for changes in buffering capacity gives a rather sensitive index of differences in buffer. It is recognized that with the technique employed it is impossible to correct for changes in cell buffer incidental to dilution of the menstruum by the added acid or alkali. Preliminary

TABLE 1a

Electrometric titration of distilled water

Acid portion of the curve

OBSERVED E.M.F.	pH	ACID ADDED (ACCUMULATIVE)	ACID ADDED	LOG OF GRAMS OF ACID ADDED	LOG OF GRAMS OF ACID ADDED
		N/500			
volts		*cc.*	*grams*		
0.560	5.35	0.0	0.0000000		$-\infty$
0.534	4.91	0.1	0.0000073	$\bar{6}.86$	−5.14
0.513	4.55	0.3	0.0000219	$\bar{5}.34$	−4.66
0.496	4.27	0.65	0.0000475	$\bar{5}.68$	−4.32
0.485	4.07	1.0	0.0000730	$\bar{5}.86$	−4.14
0.470	3.82	2.0	0.0001460	4.16	−3.84
		N/5			
0.428	3.10	0.1	0.0008760	$\bar{4}.94$	−3.06
0.411	2.80	0.3	0.0023360	$\bar{3}.37$	−2.63
0.398	2.59	0.6	0.0045260	$\bar{3}.66$	−2.34
0.375	2.20	1.5	0.0110960	2.04	−1.96

Alkaline portion of the curve

OBSERVED E.M.F.	pH	ALKALI ADDED (ACCUMULATIVE)	ALKALI ADDED	LOG OF GRAMS OF ALKALI ADDED	LOG OF GRAMS OF ALKALI ADDED
		N/500			
volts		*cc.*	*grams*		
0.560	5.35	0.0	0.000000		$-\infty$
0.738	8.37	0.25	0.000020	$\bar{5}.30$	−4.70
0.786	9.17	0.55	0.000044	$\bar{5}.64$	−4.36
0.822	9.78	1.10	0.000088	$\bar{5}.94$	−4.06
0.851	10.27	2.00	0.000160	4.20	−3.80
		N/5			
0.898	11.06	0.20	0.001760	$\bar{3}.25$	−2.75
0.927	11.53	0.50	0.004160	$\bar{3}.62$	−2.38
0.951	11.94	1.05	0.008560	$\bar{3}.93$	−2.07

Twenty cubic centimeters of fluid titrated in each chamber.

experiments demonstrated, however, that these were not significant because the measurable buffer is independent of cell concentration within the limits of change which occur with our technique. We may recapitulate the definition of buffer ratio in the following manner:

$$\text{Buffer ratio} = \frac{\text{grams acid or alkali to shift pH } (a \text{ to } b) \text{ in bacterial suspension}}{\text{grams acid or alkali to shift pH } (a \text{ to } b) \text{ in suspension fluid}}$$

IV. GENERAL PROCEDURE

The procedure was to use sixteen to twenty-four hour old cultures of *Bact. coli* of the same strain which had been used to study viability, grown at 37°C. on nutrient agar in Kolle flasks. The growth was washed off with distilled water and

TABLE 1b

Electrometric titration of Bact. coli in distilled water

Acid portion of the curve

OBSERVED E.M.F.	pH	ACID ADDED (ACCUMULATIVE)	ACID ADDED	LOG OF GRAMS OF ACID ADDED	LOG OF GRAMS OF ACID ADDED
		N/500			
volts		*cc.*	*grams*		
0.647	6.83	0.0	0.000000		$-\infty$
0.642	6.75	0.1	0.0000073	$\bar{6}.86$	−5.14
0.588	5.72	0.5	0.0000365	$\bar{5}.56$	−4.44
0.491	4.17	1.5	0.0001095	$\bar{4}.04$	−3.96
0.461	3.67	3.0	0.0002190	$\bar{4}.34$	−3.66
		N/5			
0.406	2.72	0.1	0.0009490	$\bar{4}.98$	−3.02
0.360	1.95	1.0	0.0075190	$\bar{3}.88$	−2.12

Alkaline portion of the curve

OBSERVED E.M.F.	pH	ALKALI ADDED (ACCUMULATIVE)	ALKALI ADDED	LOG OF GRAMS OF ALKALI ADDED	LOG OF GRAMS OF ALKALI ADDED
		N/500			
volts		*cc.*	*grams*		
0.645	6.80	0.0	0.000000		$-\infty$
0.652	6.92	0.1	0.000008	$\bar{6}.90$	−5.10
0.705	7.81	0.5	0.000040	$\bar{5}.60$	−4.40
0.808	9.55	1.5	0.000120	$\bar{4}.08$	−3.92
0.821	9.77	3.0	0.000240	$\bar{4}.38$	−3.62
		N/5			
0.893	10.97	0.1	0.001040	$\bar{3}.04$	−2.96
0.959	12.08	2.0	0.016240	$\bar{2}.21$	−1.79

the resulting suspension was washed three times by centrifugalization at high speed. The work of Loeb (1922) suggests that it would have been desirable to have washed the organisms and to have started the titration curves at the isoelectric point of *Bact. coli*. This was not done primarily because we were desirous of finding, if possible, how living cells react

TABLE Ic

Titration curve of distilled water. Derivative data

pH	LOG OF GRAMS PRESENT	LOG OF GRAMS PRESENT	PRESENT	ADDED
		Acid portion		
			grams	*grams*
5	−5.25	$\bar{6}.75$	0.0000056	
5–4				0.000083
4	−4.05	$\bar{5}.95$	0.000089	
4–3				0.0012
3	−2.90	$\bar{3}.10$	0.0013	
3–2				0.019
2	−1.70	$\bar{2}.30$	0.020	
		Alkaline portion		
6	−5.72	$\bar{6}.28$	0.0000019	
6–7				0.0000033
7	−5.28	$\bar{6}.72$	0.0000052	
7–8				0.000010
8	−4.83	$\bar{5}.17$	0.000015	
8–9				0.000025
9	−4.40	$\bar{5}.60$	0.000040	
9–10				0.00008
10	−3.93	$\bar{4}.07$	0.00012	
10–11				0.0013
11	−2.85	$\bar{3}.15$	0.0014	
11–12				0.0075
12	−2.05	$\bar{2}.95$	0.0089	

to the changes mentioned below. When salt solutions were used as the menstrua, the bacteria were washed twice in water, once in the salt solution to be studied and were finally suspended in the salt solution. The titrations were started immediately when salts were used. But in the case of those experiments in which water was used for the dispersion medium,

the suspension were often left on ice over night for the purpose of getting the material buffered to as near neutrality as possible. The salt solutions were all adjusted to between pH 6 and 7 before seeding because of the importance of the initial pH on the viability of the cells. Tables 1a, 1b, 1c, cd, 1d, and figure 2 are introduced to illustrate the experimental and de-

TABLE 1d

Buffer ratios of Bact. coli in distilled water

pH	LOG OF GRAMS PRESENT	LOG OF GRAMS PRESENT	PRESENT	ADDED	BUFFER RATIO
Acid portion					
			grams	*grams*	
6	−4.62	5.38	0.000024		
6–5				0.000039	
5	−4.20	5.80	0.000063		
5–4				0.00008	0.96
4	−3.85	4.15	0.00014		
4–3				0.00049	0.41
3	−3.20	4.80	0.00063		
3–2				0.0056	0.30
2	−2.21	3.79	0.0062		
Alkaline portion					
7	−5.05	6.95	0.0000089		
7–8				0.000036	3.60
8	−4.35	5.65	0.000045		
8–9				0.000040	1.60
9	−4.07	5.93	0.000085		
9–10				0.00023	2.88
10	−3.50	4.50	0.00032		
10–11				0.0008	0.62
11	−2.95	3.05	0.0011		
11–12				0.012	1.60
12	−1.90	2.10	0.013		

rivative data of a representative experiment. Figure 3 illustrates the case when no buffering capacity is present.

V. RESULTS OF EXPERIMENTS

The materials first used as menstrua for these studies were water, the two concentrations of electrolytes which had been found to be just toxic in the experiments of Winslow and Falk, viz., 0.725 M NaCl and 0.145 M $CaCl_2$, and a mixture

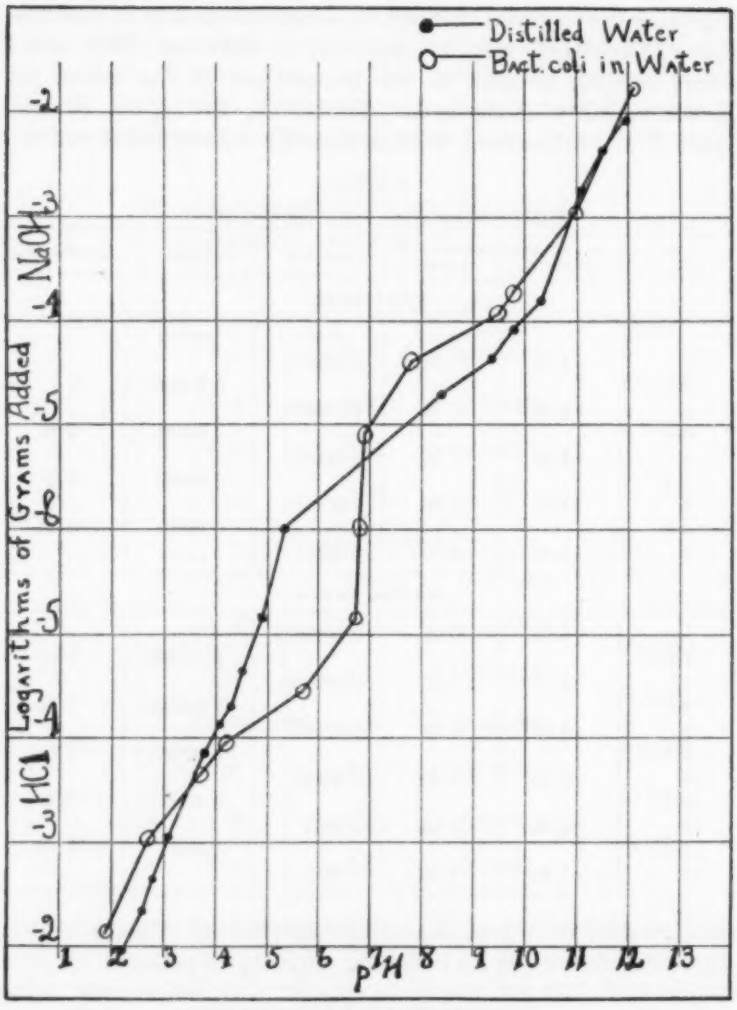

FIG. 2. TITRATION CURVES OF DISTILLED WATER AND OF BACT. COLI IN DISTILLED WATER

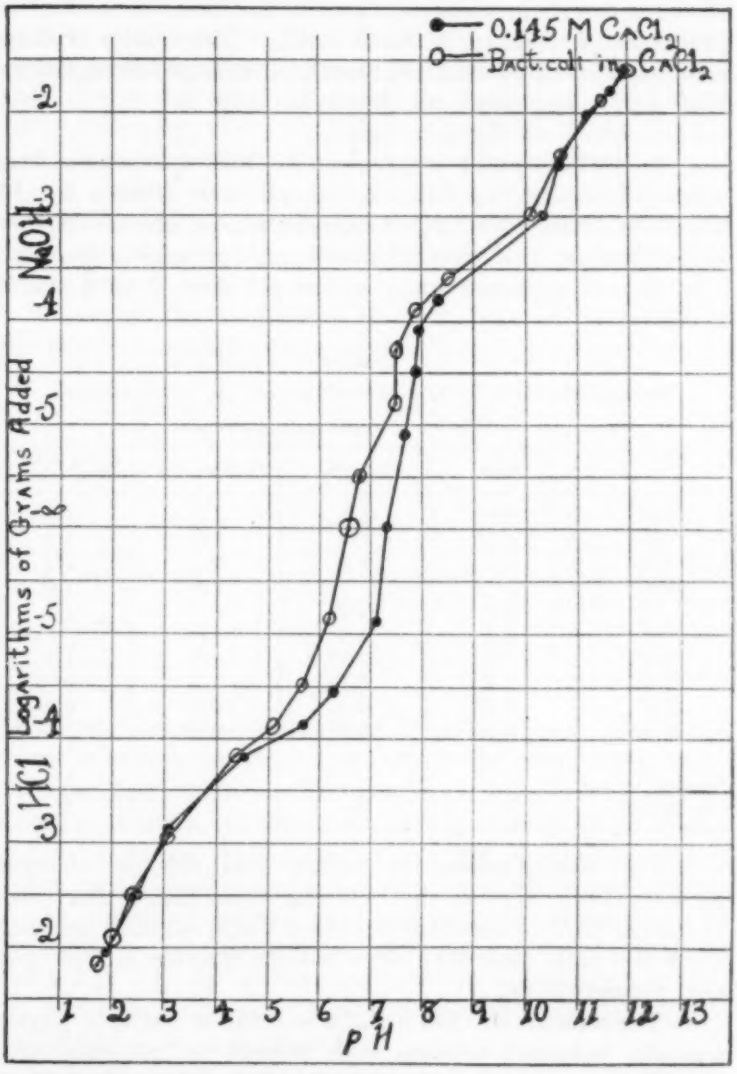

FIG. 3. TITRATION CURVES OF 0.145 M $CaCl_2$ AND OF BACT. COLI IN 0.145 M $CaCl_2$

of the two salts in which antagonism had been demonstrated, i.e., 0.580 M Nacl + 0.145 M $CaCl_2$. The results of these experiments were summarized elsewhere (Falk and Shaughnessy, 1922–1923), somewhat as shown in table 2.

It appears from the data that:

1. In distilled water suspensions of *Bact. coli* possess very marked buffering capacities in the pH zone from 4 to 10. These capacities are strongest near the zone of greatest physiological interest, i.e., from pH 6 to 7, are appreciable from pH 4 to 10, and approach unity at the pH zones 3 to 4 and 10 to 11.

TABLE 2

Average buffer ratios for Bacterium coli in water and in salt solutions

pH zone	Ratio for Bact. coli in:			
	Water	0.725 M NaCl	0.145 M $CaCl_2$	0.580 M Na Cl + 0.145 M $CaCl_2$
3–2	0.85	1.5	1.0	1.2
4–3	0.77	1.0	1.0	1.3
5–4	1.7	1.8	0.75	1.4
6–5			1.0	1.0
7–6	8.2	1.9		1.3
7–8	4.7	1.4		
8–9	1.7	0.75	1.2	1.3
9–10	2.4	0.88	1.5	0.75
10–11	0.59	0.92	0.92	0.91
11–12	0.93	0.68	0.91	1.0
Number of experiments.....	7	3	3	4

2. The concentrations of sodium and calcium chlorides used markedly depress the buffering capacities of *Bact. coli*. It is significant to note that a 0.145 M $CaCl_2$ solution depresses the buffer ratio more than does sodium chloride in five times that concentration.

3. The solution of 0.580 M NaCl + 0.145 M $CaCl_2$ (a physiologically balanced solution with respect to influence upon viability) acts upon buffer capacity like the unmixed $CaCl_2$ solution.

4. The acidic pH values at which the buffering capacity of *Bact. coli* becomes insignificant are approximately those at which this organism is known to be spontaneously agglutinable and to be isoelectric with the menstruum (Michaelis, 1911; Eisenberg, 1919; Northrop and DeKruif, 1922; Winslow, Falk and Caulfield, 1923). It is therefore significant to note that a similar reduction in the buffer ratio is attained at alkaline as well as at acidic reactions. This observation suggests the existence of a second—an alkaline—isoelectric point for bacteria.

It seemed that logically the next step should be to try the effect of varying the concentrations of the two salts used.

TABLE 3

SOLUTION	NUMBER OF EXPERIMENTS	BUFFER RATIOS IN pH ZONE									
		2 to 2.9	3 to 3.9	4 to 4.9	5 to 5.9	6 to 6.9	7 to 7.9	8 to 8.9	9 to 9.9	10 to 10.9	11 to 11.9
Distilled water	7	0.50	1.22	**3.47**	**7.88**	**12.27**	**9.60**	**3.90**	**4.34**	0.78	0.53
0.0725 M NaCl	3	0.89	**2.83**	**3.07**	**4.47**		**3.88**	**2.42**	1.47	1.00	1.13
0.145 M NaCl	3	1.00	1.11	**1.73**	**2.12**	**3.77**	**1.92**	**1.64**	1.00	0.56	0.81
0.3625 M NaCl	1	1.27	1.46	**1.76**			**2.65**	1.02	0.76	1.00	1.22
0.725 M NaCl	3	1.45	1.00	**1.78**		**1.87**	1.40	0.75	0.88	0.92	0.68
1.450 M NaCl	5	1.27	1.28	**2.03**	**1.82**		**2.59**	**1.85**	**1.59**	0.96	0.87

The buffer ratios were calculated as before, using the titration curve of each concentration of salt for the basis of the buffer ratio for that molarity. The results of the sodium series are shown in table 3 and figure 4. It has seemed to us that it would be useless to consider any slight divergence from a buffer ratio of 1.00 as significant because of the errors inherent in the biological material and perhaps to a lesser degree in the method. (A difference of 0.2 of a pH unit as between 6.0 and 6.2 or 6.8. and 7.0 is equivalent to a difference of 37.5 per cent in hydrogen ion concentration because of the logarithmic nature of the pH symbols.)

With due regard to the errors in the material and method, we have arbitrarily set a deviation of 0.50 from the ratio of unity as significant. In tables 3 and 4 all values which

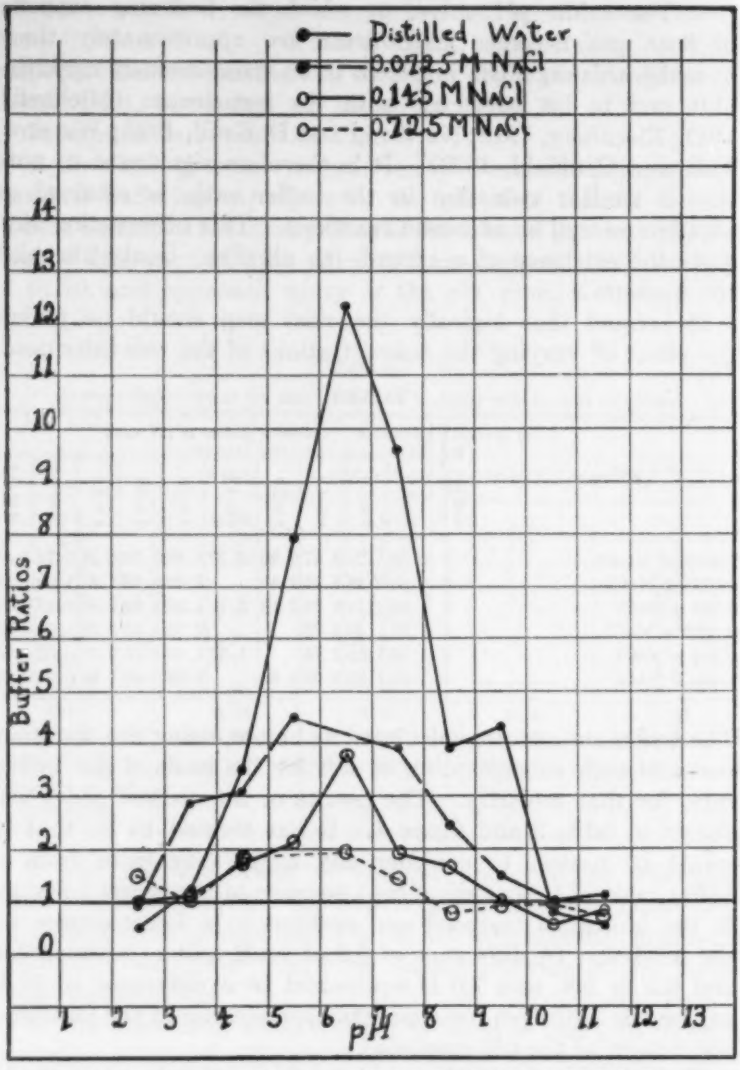

FIG. 4. AVERAGE BUFFER RATIOS OF BACT. COLI IN DISTILLED WATER AND IN SODIUM CHLORIDE SOLUTIONS

exceed 1.50 are set in boldface type. The water curve figures are presented for comparative purposes. From the table for each experiment a series of curves was drawn, plotting the ratios for each pH zone for the more important concentrations and for water. The data presented in these tables are averages calculated from seven experiments with suspensions in water, fifteen in sodium chloride, and twelve in calcium chloride solutions of various concentrations, and from four with suspensions in a solution containing both salts. From these values it seems to us the following conclusions may be drawn:

1. In water there is an appreciable buffer from pH 4 to 10 with a peak between 6 and 7 and with fairly high values between 5 and 8.

2. In the most dilute solution of sodium chloride (0.0725 M), there is a depression of the buffer at all pH values, but there seems to be an appreciable capacity to unite with hydrogen and hydroxyl ions from pH 3 to 10 (although the deviation from 1.50 at pH 9 to 9.9 is very slight). The broadening of the zone of buffer on the acid side perhaps points to something significant because it has been found that sodium chloride broadens the curve of velocity of electrical migration of *Bact. coli* in a concentration similar to this.

3. From the concentrations of 0.145 M NaCl ("isotonic") to 0.725 M NaCl the area of buffer progressively becomes smaller and smaller until at the latter concentration it is present only in the limited zone from pH 4 to 7. It may be noted that the loss in breadth of the area has been practically all on the alkaline side of neutrality (pH 7.0). Attention may be called also to the fact that as the zone becomes narrower, the significant ratios become smaller.

4. A concentration of 1.450 M NaCl (toxic enough to kill practically all bacteria in from three to six hours) produces what at first seems a surprising result, i.e., both a broadening of the range of significant buffer to the original pH 4 to 10 and an increasing of the individual ratios at the zones of buffer until they are almost as great as in the least toxic

concentrations. This finding can probably be explained by assuming that this concentration of salt (which is 10× isotonic) may produce liberation of proteins (either by lysis or by extrusion of amphoteric products) which unpublished data on autolyzed *Diplococcus pneumoniae* demonstrate to have higher buffering powers than the organized cell.

The results obtained by varying the concentration of $CaCl_2$ from 0.00725 M $CaCl_2$ to 0.290 M $CaCl_2$ are shown in table 4 and in figure 5. Boldface type is used again to show the buffer ratios which seem to be significant.

TABLE 4

SOLUTION	NUMBER OF EXPERIMENTS	BUFFER RATIO IN pH ZONE									
		2 to 2.9	3 to 3.9	4 to 4.9	5 to 5.9	6 to 6.9	7 to 7.9	8 to 8.9	9 to 9.9	10 to 10.9	11 to 11.9
Distilled water	7	0.50	1.22	**3.47**	**7.88**	**12.27**	**9.60**	**3.90**	**4.34**	0.78	0.53
0.00725 M $CaCl_2$	2	1.21	1.21	1.44	**1.76**		0.82	0.53	1.00	0.84	0.93
0.0145 M $CaCl_2$	1	1.00	1.10	1.39		1.14	0.91	1.38	1.20	1.40	1.20
0.0725 M $CaCl_2$	1		1.47	**1.57**	1.29		**1.70**	1.44	0.89	0.81	0.85
0.145 M $CaCl_2$	3	1.04	1.04	0.75	1.02			1.20	1.46	0.92	0.91
0.290 M $CaCl_2$	5	1.23	1.05	1.37	**2.06**		**3.22**	**2.40**	**1.49**	1.21	0.92
0.580 M NaCl + 0.145 M $CaCl_2$	4	1.21	1.27	1.38	1.00	1.32		1.32	0.75	0.91	1.00

These findings may be summarized as follows:

1. The only concentrations of $CaCl_2$ which seem to show buffer are the 0.00725 M $CaCl_2$ at pH 5 to 6, the 0.0725 M $CaCl_2$ at 4 to 5 and 7 to 8, and the 0.290 M $CaCl_2$ at from pH 5 to 9.

2. Of these concentrations the first is perhaps significant of a true buffering capacity on the acid side although it is the average of but two curves and is so narrow a zone as to be somewhat questionable.

3. The buffer values in the 0.0725 M $CaCl_2$ are questionable because they are small in value, rather widely separated and the results from but one experiment.

4. The values for buffering capacity in 0.290 M $CaCl_2$ are probably to be explained on the same basis as the values

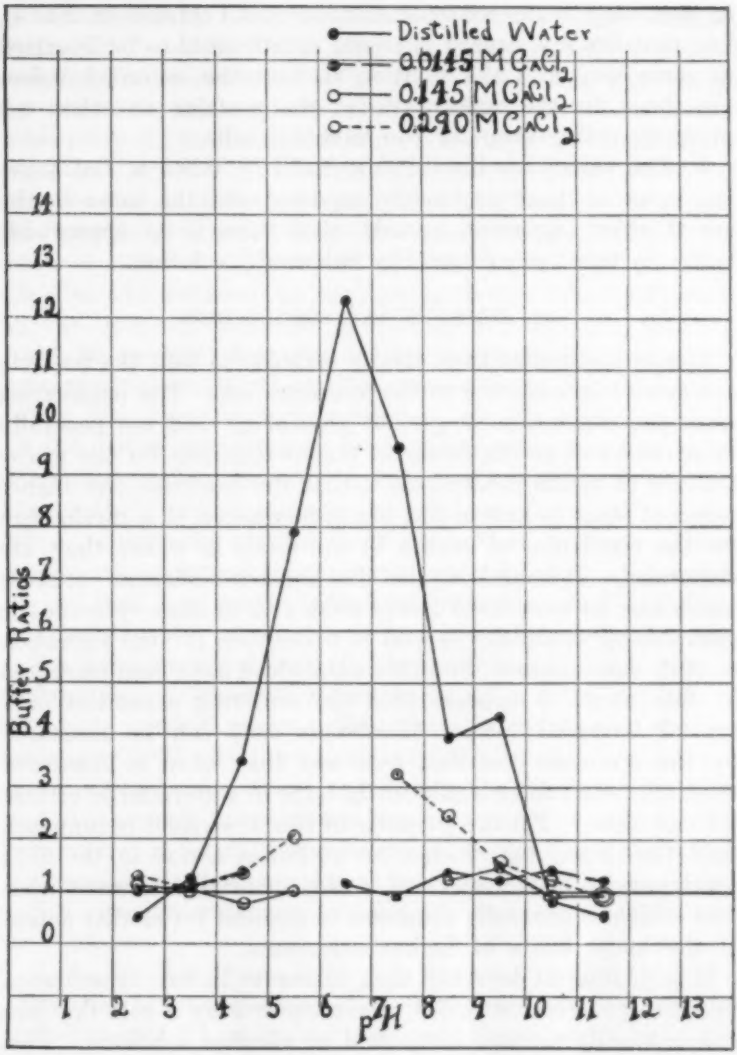

FIG. 5. AVERAGE BUFFER RATIOS OF BACT. COLI IN DISTILLED WATER AND IN CALCIUM CHLORIDE SOLUTIONS

in the very toxic concentration of NaCl (1.450 M NaCl), i.e., that the salt caused bacterial constituents to be liberated in some way into the solution so that the recorded values are those for the buffer ratio of the proteins or other cell products rather than for the bacterial cells.

5. The values for the 0.580 M NaCl + 0.145 M $CaCl_2$ are the same as those previously reported and the same results are therefore apparent, namely, that there is no appreciable buffer in this "physiologically balanced" solution.

VI. SUMMARY AND CONCLUSIONS

Numerous studies have clearly established that the bacteria are extremely sensitive to the hydrogen ion. The implication from the conclusion of general physiology and the generally accepted views on the fitness of the environment for the maintenance of viable protoplasm is that the bacteria, like higher forms of plant or animal life, are in possession of a mechanism for the regulation of acidity in the fluids in which they are suspended. It is also implied that aqueous menstrua—because water may be considered a very weak acid or base—provide the desirable opportunities as well as necessities for the operation of such mechanisms. From the data which have been presented in this paper it appears that the buffering capacities of a typical bacterial species (*Bacterium coli*) can be measured by the methods described here and that when so measured they are found to be significantly large in water and in certain salt solutions. For the purposes of this analysis it is supposed that the underlying mechanism of buffer action in the fluid contiguous to a bacterial cell is not essentially different from that which is generally proposed to account for similar action in the body fluids of higher organisms.

It is of interest to recall that whenever in our experiments the zone of effective buffer was narrowed by a salt the loss was especially marked in neutral or alkaline solutions. This observation is entirely in harmony with deductions which may be drawn from other observations on physico-chemical characteristics of the bacterial cell. Thus, it has been clearly

established (vide Northrop and DeKruif, 1922; Winslow, Falk and Caulfield, 1923) that the bacterial cell in neutral solutions is electronegative to water. Whether this charge on the cell is to be accounted for on the basis of a selective adsorption of electronegative ions or on the theory of membrane equilibria or both is immaterial for the present purposes. It remains true that the bacterial cell or its principal constituents are especially reactive with electropositive ions and (on the basis of either theory) should be the more reactive the more alkaline the solution up to certain limits. The differences in the observed behavior of sodium and calcium chlorides must be considered as due to the specific properties of the cations of these salts which dissociate to give a common anion. When the menstruum is rendered acid the eletronegativity of the cell is reduced (and is abolished when the acidity attains the specific value which is termed the "isoelectric point"). Its reactivity with cations—it may be expected—will be diminished. Hence the reactions with the sodium or calcium cations which are evidenced by depression of the buffer ratio should be, as they are, more marked in neutral or alkaline than in acid solutions.[1]

We have observed from the data which were cited that the buffer ratio is appreciably depressed by calcium chloride in non-toxic concentrations. This observation suggests that interference with the mechanism of buffer action may become physiologically significant only when the reaction of cation and cell or cell constitutents has proceeded to some specific point and that viability may be maintained although the buffering capacity has been considerably reduced or nearly abolished.

Our specific experimental findings may be recapitulated as follows:

1. In distilled water *Bact. coli* possesses distinct buffering capacities which are greatest in the most favorable zone for viability—pH 6 to 6.9—and which are significant in the range which is of physiological importance, the pH range from 4 to 10.

[1] The experiments of Stearn and Stearn (1923) suggest a similar explanation for the reaction of bacteria with acidic and basic dyes.

Above pH 10 and below pH 4 the buffer ratio approximates unity and further may be so significantly below unity as to suggest that there is actual liberation of, rather than reaction with, the hydrogen or hydroxyl ion which predominates in the surrounding medium. The existence of a second, alkaline isoelectric point is suggested because the buffer becomes insignificant several pH units away from the acid isoelectric point (which is below pH 1.0 for this strain) and likewise becomes insignificant at certain alkaline pH values.[2]

2. In sodium chloride solutions the buffering capacities were depressed with all the strengths used (0.0725 M to 1.450 M); but it seems that the breadth of the significant buffer zone and magnitudes of the individual ratios increase as the dilution (or decrease as the concentration) increases up to the point of just appreciable toxicity of the salt for the bacteria. Beyond that point the buffer is somewhat increased but is still lower than the buffer in distilled water or in very dilute sodium chloride solution. In these concentrated solutions it is probable that we are dealing with unorganized cell constituents rather than with bacterial cells.

3. In calcium chloride solutions (0.0145 M to 0.290 M) the buffering capacities were practically abolished in all the solutions which were non-toxic at favorable pH. It is possible that there is an appreciable buffer in the pH zone 5 to 5.9 in the most dilute solutions (0.00725 M). The toxic concentration showed the same increase in buffer as did the toxic concentration of sodium chloride and very probably for the same reason.

4. The "physiologically balanced" solution of NaCl + $CaCl_2$ (0.580 M + 0.145 M) showed no material increase in buffering capacities over that of the solution containing calcium chloride alone.

[2] The existence of this alkaline isoelectric (isopotential) point for bacterial cells has been demonstrated by the data recently reported by Winslow and Shaughnessy (1924).

REFERENCES

BROWN, J. H. 1921 Hydrogen ions, titration and the buffer index of bacteriological media. Jour. Bact., **6**, 555.

CLARK, W. M. 1920, 1922 The Determination of Hydrogen Ions. Baltimore.

COHN, E. J., GROSS, J. AND JOHNSON, O. C. 1919–1920 The isoelectric points of the proteins in certain vegetable juices. Jour. Gen. Physiol., **2**, 145.

COHN, E. J., WOLBACH, S. B., HENDERSON, L. J. AND CATHCART, P. H. 1918–1919 On the control of rope in bread. Jour. Gen. Physiol., **1**, 221.

EISENBERG, P. 1919 Ueber Säureagglutination von Bakterien und über chemische Agglutination im allgemeinen. Centr. Bakt., **83**, 70, 472 561.

FALK, I. S., AND SHAUGHNESSY, H. J. 1922–1923 Effect of certain electrolytes on the buffering power of *Bacterium coli*. Proc. Soc. Exp. Biol. and Med., **20**, 426.

LOEB, J. 1922 Proteins and the theory of colloidal behavior. New York, 292 pp.

MICHAELIS, L. 1911 Die Säureagglutination der Bakterien, unsbesondere der Typhusbazillen. Deut. med. Wochenschr., **37**, 969.

NORTHROP, J. H., AND DEKRUIF, P. 1922 The stability of bacterial suspensions. II. The agglutination of the bacillus of rabbit septicemia and of *Bacillus typhosus* by electrolytes. Jour. Gen. Physiol., **4**, 639.

STEARN, E. W., AND STEARN, A. E. 1923 The mechanical behavior of dyes, especially gentian violet, in bacteriological media. Jour. Bact., **8**, 567.

WINSLOW, C.-E.A., AND FALK, I. S. 1923 Studies on salt action. VIII. The influence of calcium and sodium salts at various hydrogen ion concentrations upon the viability of *Bacterium coli*. Jour. Bact., **8**, 215.

WINSLOW, C.-E.A., FALK, I. S., AND CAULFIELD, M. F. 1923 Electrophoresis of bacteria as influenced by hydrogen ion concentration and the presence of sodium and calcium salts. Jour. Gen. Physiol., **6**, 177.

WINSLOW, C.-E. A. AND SHAUGHNESSY, H. J. 1924 The alkaline isopotential point of the bacterial cell. Jour. Gen. Physiol., **6**, 697.

Pag
Mi's

ge

ssing

THE EFFECT OF ENVIRONMENTAL CHANGES ON THE GROWTH, MORPHOLOGY, PHYSIOLOGY AND IMMUNOLOGICAL CHARACTERISTICS OF BACTERIUM TYPHOSUM[1]

CHARLES A. STUART

From the Bacteriological Laboratories of Brown University, Providence, Rhode Island

Received for publication July 16, 1924

The question of just how far environmental changes will affect the cultural characters of a species or a single strain of a species is one that needs more study. A review of the literature shows considerable contradictory evidence resulting from the experiments of different investigators.

In an attempt to work out the relation existing between the growth and fermentative powers of bacteria, Herter (1909) and Jacoby (1917) came to opposite conclusions. The former concludes from his work that the fermentative power of species may be greatly impaired or entirely lost without any appreciable effect upon their growth; whereas Jacoby contends that growth and fermentative ability run a fairly parallel course.

Pacini (1918), working on the vitamine content of bacteria obtained an excellent growth on Uschinsky's medium with the Anderson strain of *Bact. typhosum*, while Damon (1921), working on the same problem and using the same medium, was unable to obtain any growth with the typhoid strain which he used.

Kirstein (1904) found that, when cultivated on synthetic medium, the typhoid bacillus became spontaneously agglutinable, while Frankel and Barker (1919) reported no change in the agglutinability of the typhoid bacterium cultivated on various synthetic media.

Joos (1902), Joachin (1904), and Scheller (1905), (1906) have come to the conclusion that heating the typhoid bacillus alters its antigenic and

[1] Thesis submitted to the Faculty of Brown University in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

agglutinating properties. Eisenberg (1906a), on the other hand, finds that the agglutinating property of bacteria is impaired by heat, but its ability to incite antibody production remains intact.

Sehrwald (1905) claims that growing *Bact. typhosum* on a potato medium increased its agglutinability, while Eisenberg (1906b) and others were unable to substantiate his claim.

Several investigators (1923) have cultivated inagglutinable bacilli from the spleen, gall bladder and urine of typhoid patients. But Porges and Prantschoff (1906) were unable to obtain the same results from experimental animals.

One thing at least may be offered as a partial explanation of these discrepancies. For the most part the foregoing experiments have been carried out with a single strain of the species and there has been too great a tendency to interpret the results as applying to the species in general.

The following plan suggested itself as one which would show how far environmental influences would affect a single strain of a species, and to what degree we could interpret the modifications as being indicative for the whole species. Several strains of typhoid bacillus were selected, some from analogous sources and at varying intervals of time, while others were isolated from widely different sources. From this number one strain, typical in every respect, was grown on different media and at different temperatures. Its growth, morphology, physiology and immunological variations were noted in detail. The different media used have a wide range in nutritive value. One just supported growth, while another was of exceptional nutritive qualities. Beef extract agar was used as a control. Finally the selected strain was grown in as wide a range of temperatures as possible. The remaining strains were studied for variation in as many ways as circumstances would permit, without carrying through the whole procedure as outlined for the selected strain.

THE STRAINS SELECTED FOR THE EXPERIMENT

Strain I isolated from feces nine months previous to the experiment.
Strain II isolated from feces five months previous to the experiment.

Strain III isolated from feces five months previous to the experiment.

Strain IV isolated from feces three months previous to the experiment.

All the foregoing cultures were isolated on Endo's medium, and transferred to Russell's double sugar (litmus indicator); finally to agar on which they were carried by weekly transplants until used in the experiments.

Strain V obtained from feces eighteen months previous to the experiment.

Isolated on eosin methylene blue agar from a typhoid carrier; transferred to agar and carried in semi-monthly transplants.

Strain VI obtained from feces three years previous to the experiment.

Isolated on brilliant green agar and used as a laboratory stock culture in class work for over two years in which no careful record was kept of its transfers. It was passed through animals at least three times, cultivated in immune serum, and carried in glucose broth for six months.

Strain VII procured from blood nine months previous to the experiment.

Strain VIII procured from blood five months previous to the experiment.

Isolated on eosin methylene blue agar and carried on agar with weekly transplants. VIII showed characteristic inagglutinability on isolation, but this property was recovered some time before the start of the work.

Strain IX obtained from urine eight weeks previous to the experiment.

Isolated on Endo's medium, transferred to lead acetate and carried in weekly transplants on agar.

Strain X, obtained from bile several days previous to the experiment.

Isolated on eosin methylene blue agar from a chronic carrier.

THE PHYSIOLOGY OF THE STRAINS SELECTED

In order accurately to establish the physiological status of all strains previous to any modification in their envrionment, and to select a strain typical in all respects, the cultures were were grown in the following sugar broths: arabinose, xylose, rhamnose, glucose, levulose, galactose, maltose, lactose, sucrose, raffinose, dextrin, inulin, manitol, dulcitol, and glycerol. They were examined for motility, tested for proteolytic enzymes in gelatin, for acid ratio in the Voges Proskauer broth, for the production of hydrogen sulphide in lead acetate, and for indol, and their reaction noted in litmus milk.

All sugar broths were made in the following manner: A twenty-four hour veal infusion filtrate was inoculated with a culture of *Bact. coli* and placed in the incubator for thirty-six hours; purified talc was then added and the mixture filtered through paper pulp; 1 per cent Witte's peptone and 0.5 per cent sodium chloride were added and autoclaved into solution for ten minutes at 15 pounds pressure. From this point all manipulations were carried out in sterile containers. The broth was adjusted to the indicator, divided into appropriate amounts and a one per cent solution of the various sugars dissolved in sterile distilled water was added. The media were distributed in test tubes previously sterilized and the whole sterilized for ten minutes at 10 pounds pressure.

By this method fractional sterilization is eliminated, spore contamination is reduced to a minimum, and previous sterilization of the sugars under low pressure and laboriously tubing the completed media with absolute aseptic precautions is unnecessary. Inulin alone failed to give a sterile medium, and as a spore-forming rod was isolated from two lots of this substance the medium was made in the usual way and sterilized for fifteen minutes at 15 pounds. The indicator used in all sugar broths was a phenol red-china blue solution devised by Morishima (1920).

The media used for motility, proteolytic enzymes and the methyl-red test were plain broth, gelatin, and glucose potassium phosphate broth, made up according to standard methods. Motility was determined at the end of eighteen hours. The gelatin stick cultures were grown at room temperature and examined weekly for six weeks. The Voges-Proskauer reaction was determined at the end of forty-eight hours. The medium used for the detection of hydrogen sulphide was Difco's dehydrated lead acetate agar (reference number 9709), all cultures were grown for eight days and examined every twelve hours. The test for indol was made after growing the organisms for seventy-two hours in Witte's peptone broth. Nelson's (1916) vanillin test was used with concentrated hydrochloric acid. A delicacy of one part in five million is claimed for this particular test. A powdered litmus milk medium devised by Hamilton (1921) was employed for testing the reaction of the different strains in milk. Cultures were carried in this medium for two weeks and examined daily.

The ten strains were cultivated on agar slants for five successive days, transplanted to broth, and at the end of eighteen hours a standard loopfull of the broth was transferred to the sugar and special test broth. Gelatin and lead acetate agar

were inoculated from the last agar transplant. The sugar broths were examined daily for three weeks, the other media as specified, and the results tabulated in chart 1.

The chart in general shows a few peculiarities on the part of the individual strains. Strains I and IV belong to the so-called non-xylose fermenting group, two such out of ten being an unusually high percentage. Both strains were repeatedly trans-

MEDIA	STRAINS									
	I	II	III	IV	V	VI	VII	VIII	IX	X
Arabinose	0	0	0	0	0	0	0	0	0	0
Xylose	0	A	A	0	A	A	A	A	A	A
Rhamnose	0	0	0	0	0	0	0	0	0	0
Glucose	A	A	A	A	A	A	A	A	A	A
Levulose	A	A	A	A	A	A	A	A	A	A
Galactose	A	A	A	A	A	A	A	A	A	A
Maltose	A	A	A	A	A	A	A	A	A	A
Lactose	0	0	0	0	0	0	0	0	0	0
Sucrose	0	0	0	0	0	0	0	0	0	0
Raffinose	0	A	0	0	0	0	0	0	0	0
Dextrin	A	A	A	A	A	A	A	A	A	A
Inulin	0	0	0	0	0	0	0	0	0	0
Manitol	A	A	A	A	A	A	A	A	A	A
Dulcitol	A	A	0	A	A	0	0	0	A	A
Glycerol	A	A	A	A	A	A	A	A	A	0
Gelatin	0	0	0	0	0	0	0	0	0	0
Methyl-red	P	P	P	P	P	P	P	P	P	P
Lead-acetate	P	0	P	P	P	P	P	P	P	0
Indol	0	0	0	0	0	0	0	0	0	?
Litmus-milk	A	A	A	A	A	A	A	A	A	Alk
Motility	Sl	P	P	Sl	P	P	P	P	P	P

CHART 1. THE PHYSIOLOGICAL CHARACTERISTICS OF THE TEN STRAINS SELECTED FOR THE EXPERIMENT

A, acid; P, positive; Alk, alkaline; Sl, slight

ferred in xylose broth. After fourteen transplants IV became a rapid (twenty-four hour) xylose fermenter, while I did not respond until the twenty-second transplant.

Strain II produced acid in raffinose, a power which ordinarily the typhoid bacillus does not possess. The reactions of the strains in dulcitol were not consistent, some strains producing acid on an initial transplant but not in a subsequent transfer.

In all cases where the blackening of lead acetate acetate occurred it was very slight. Some strains produced only a black spot on the surface of the medium at the point of inoculation, and none along the line of puncture. Others produced merely a suggestion of discoloration along the line of puncture. Strains II and X, however, failed to produce any discoloration whatever, even after successive transplants in this medium. Strains I and IV were decidedly sluggish in their motility and no amount of transplanting served to bring this factor up to the standard set by the other eight strains.

GROWTH OF THE STRAINS ON MEDIA OF HIGH AND LOW NITRITIVE VALUE AND AT HIGH AND LOW TEMPERATURES

The next step in the procedure was to find one medium on which growth was not as good as that on standard agar, and one on which growth was much better than on standard agar. Synthetic media were naturally the choice for the first, and the work of Dolt (1908) Doryland (1926) and others was reviewed. As the exact composition of the medium was of no concern, the various kinds given by Dolt were selected for trial because of their simplicity.

The following types of media were tried:

1	Asparagin	1.0 gram
	Sodium nitrate	0.2 gram
	Agar solution	100.0 cc.
2	Asparagin	1.0 gram
	Potassium nitrate	0.2 gram
	Agar solution	100.0 cc.
3	Asparagin	1.0 gram
	Ammonium nitrate	0.2 gram
	Agar solution	100.0 cc.
4	Asparagin	1.0 gram
	Sodium phosphate	0.2 gram
	Agar solution	100.0 cc.
5	Asparagin	1.0 gram
	Potassium phosphate	0.2 gram
	Agar solution	100.0 cc.

6	Asparagin	1.0 gram
	Ammonium phosphate	0.2 gram
	Agar solution	100.0 cc.
7	Asparagin	0.4 gram
	Ammonium lactate	0.6 gram
	Sodium phosphate	0.2 gram
	Sodium chloride	0.5 gram
	Agar solution	100.0 cc.
8	Asparagin	0.34 gram
	Ammonium lactate	1.00 gram
	Sodium chloride	0.50 gram
	Magnesium sulphate	0.02 gram
	Calcium chloride	0.01 gram
	Potassium phosphate	0.10 gram
	Glycerol	4.00 gram
	Agar solution	100.0 cc.
9	Glycerol	1.0 gram
	Ammonium phosphate	0.2 gram
	Agar solution	100.0 cc.
10	Ammonium lactate	1.0 gram
	Sodium phosphate	0.2 gram
	Agar solution	100.0 cc.
11	Malic acid	1.0 gram
	Ammonium lactate	0.2 gram
	Agar solution	100.0 cc.

The agar used throughout these experiments was washed according to the method of Ayres, Mudge and Rupp (1920). The reaction in all cases was adjusted to pH 7.0 to 7.2 with phenol red and sterilized along with control tubes containing the indicator to detect any shift in the hydrogen ion concentration due to the heat.

VARIATIONS IN THE GROWTH OF THE TEN STRAINS ON THE VARIOUS TYPES OF SYNTHETIC MEDIA

All strains were inoculated with a standard loop from an eighteen hour broth culture on all the media and incubated for two weeks at 37°C., with daily observations. As a control a laboratory stock culture was planted on standard agar in the same way. The growth on the control tube was taken as the unit 10 and all growths on synthetic agar compared with it and rated according to the amount of growth. Thus an

MEDIA	STRAIN I					STRAIN II					STRAIN III					STRAIN IV					STRAIN V				
	1 day	3 days	7 days	10 days	14 days	1 day	3 days	7 days	10 days	14 days	1 day	3 days	7 days	10 days	14 days	1 day	3 days	7 days	10 days	14 days	1 day	3 days	7 days	10 days	14 days
1					0					0					0					0					0
2					0					0					0					0					0
3					0					0					P					0					0
4					0				P					P						0					0
5					0					0					P					0					0
6					0				P					P						0					P
7					0			P						P						0				P	
8					?		P						P							0				P	
9					0				P					P						0					?
10					0				P					P						0					P
11					0					?					0					0					0
	STRAIN VI					STRAIN VII					STRAIN VIII					STRAIN IX					STRAIN X				
1				P						0					0					0					0
2					P					0					0					0					?
3			P							P					?					P				P	
4		P								?					0					?					0
5				P						0					0					P					0
6				P						P					P				P					P	
7		P							P					P				P						P	
8	P								P					P						P			P		
9			P							?					0					P				P	
10		P								P					P					P			P		
11				P						0					?					?				P	

CHART 2. SHOWING THE FIRST APPEARANCE OF GROWTH FROM THE DIFFERENT STRAINS ON ONE TRANSPLANT TO THE VARIOUS TYPES OF SYNTHETIC MEDIA

P, growth, 0, no growth

MEDIA	STRAIN I							STRAIN II							STRAIN III							STRAIN IV						
	1 transplant	2 transplante	3 transplante	5 transplante	7 transplante			1 transplant	2 transplante	3 transplante	5 transplante	7 transplante			1 transplant	2 transplante	3 transplante	5 transplante	7 transplante			1 transplant	2 transplante	3 transplante	5 transplante	7 transplante		
1	0							0							−1	−1						0						
2	0							0							0							0						
3	0							0							−1	?						0						
4	0							−1	−1						2	−1	−1					0						
5	0							?	0						−1	−1						0						
6	0							−1	0						1	?						0						
7	0							1	−1						−1	−1						0						
8	?	0						2	1	1	2	2			2	2	2	2	0			0						
9	0							1	1	0	2	2			−1	−1	1	1	0			0						
10	0							−1	−1	−1	1	1			1	1	1	1	0			0						
11	0							?	0	0	1	2			1	1	1	1	0			0						

MEDIA	STRAIN VI							STRAIN VII							STRAIN VIII							STRAIN IX						
	1 transplant	2 transplante	3 transplante	5 transplante	7 transplante			1 transplant	2 transplante	3 transplante	5 transplante	7 transplante			1 transplant	2 transplante	3 transplante	5 transplante	7 transplante			1 transplant	2 transplante	3 transplante	5 transplante	7 transplante		
1	−1	−1	−1	0	−1			0							0							0						
2	−1	−1	−1	−1	−1			0							0							0						
3	2	−1	−1	−1	2			−1	−1	0					?							−1						
4	1	−1	1	3	3			?	−1	−1	?	0			0							−1						
5	1	1	1	2	1			0	−1	1					0							−1						
6	−1	2	2	1	2			−1	0	1	?	0			1	0						1	0					
7	2	3	3	3	3			−1	−1	−1	−1	1			1	−1						1	−1					
8	3	3	3	4	4			1	1	1					−1							1	−1					
9	4	1	1	1	1			?	0	−1	0				0							1	−1					
10	5	3	3	4	4			−1	−1	?	0				−1	0						1	−1					
11	5	5	1	2	2			?	0						?	−1						1	−1					

MEDIA	STRAIN X							STRAIN XI						
	1 transplant	2 transplante	3 transplante	5 transplante	7 transplante			1 transplant	2 transplante	3 transplante	5 transplante	7 transplante		
1	0							0						
2	?							0						
3	1	−1	−1	−1	−1			−1	−1					
4	2	1	2	2	3			0	0					
5	0							0	0					
6	1	−1	−1	0	2			0	0					
7	1	1	1	1	2			0	0					
8	3	2	2	2	2			1	1	1				
9	1	−1	−1	1	2			0	?	0				
10	2	2	1	1	2			0	0	0				
11	1	−1	−1	1	1			0	0	0				

CHART 3. SHOWING THE ABILITY OF THE TEN STRAINS TO GROW ON SUCCESSIVE TRANSPLANTS ON THE VARIOUS TYPES OF SYNTHETIC MEDIA

experimental tube showing one-half the growth of the control would be rated as 5, one showing one-tenth the growth of the control as 1, and so on. Any growth less than one is reported as −1. This method gives only approximate results but is sufficiently accurate for the purpose of these experiments.

It was found in the actual experiment that there was considerable difference in the first appearance of growth from the various strains. A certain strain would appear to thrive nicely on a primary transplant, while a second or third transfer on the same medium failed to give any growth. In order to bring out this difference more clearly all results were tabulated in two ways: Chart 2 shows the time relations that existed between the first appearance of growth on the different media, and chart 3 shows the ability of the strains to thrive after successive transplants on the same medium.

The different strains show wide variations in their growth on the eleven types of synthetic media. Strains I and IV did not grow in two weeks on any medium, while VI grew on all in from one to fourteen days. Such profuse growth as that evidenced by strain VI brought up the question of contamination. The growth on media 4 and 9 was streaked out on eosin methylene blue plates. The colonies were characteristic of typhoid; they were fished and transferred to Russell's medium. Finally agglutination tests were resorted to and the results ruled out the possibility of any contamination.

It will be noted in the chart that in almost every case the growth of the initial transplant, when moderately abundant, is followed by a sharp decrease on the second transplant. Frequently the abundance of growth on the first transplant is never attained again. It would seem that from a previously favorable environment an organism gained a momentum which permitted it to flourish freely for a time in an unfavorable environment. A close examination of the chart makes one feel that this momentum is a more important factor than food requirements in determining whether a strain will survive a sudden change. However a great deal more evidence would be required to make such a claim valid, but it opens up a new field of investigation.

Through an error in transplanting a series of tubes it was discovered that when fifteen transplants of strain I (reported as negative in charts 2 and 3) were made on medium 8, one or more tubes might show growth. The growth, however, was always in the form of a few individual colonies scattered along the line of inoculation. After the largest of these colonies had been selected and transferred for several generations, uniform growth would result along the line of inoculation.

It seems that of the entire number of bacteria transferred in the initial transplant only a few possessed the ability to thrive, which would account for the appearance of single colonies along the line of inoculation. This variation in the individual members of a strain might explain why growth occurs in only one of several transplants made from the same culture, and also why one investigator obtains positive results in a certain experiment while a repetition of the experiment by another investigator gives negative results.

Strain III was selected to be studied in detail since its physiological reactions were typical in every respect and it grew fairly well on synthetic media. Of the various types of synthetic media 10 was selected for use throughout the experiment.

For the purpose of finding a medium of exceptional nutritive value the following were tried: a 50 per cent ascitic agar, veal infusion agar, and peptic digest of liver with and without Witte's peptone. Standard agar was used as a control.

After making several comparative counts on the above types of media, using strain III, the peptic digest of liver with Witte's peptone proved to be best. It was made as follows:

One hundred and fifty grams of finely minced liver and 200 grams of that portion of the stomach lining containing the gastric glands were mixed in a liter of water containing 10 cc. of commercial hydrochloric acid, and the mixture incubated for eighteen hours in a water bath at 50°C. with occasional stirring. Biuret and tryptophane tests were made every six hours. Digestion was stopped by heating in the sterilizer in free flowing steam for ten minutes. The solution was allowed to settle over night in the ice box, and decanted. One per cent Witte's peptone was added and autoclaved into solution. One and two-tenths per cent

agar was added, the pH was adjusted to 7.0 and the agar boiled into solution. The reaction was finally adjusted to 7.2 to 7.4, the medium tubed and sterilized. Throughout the experiment a fresh lot of this medium was made every month with livers and stomachs direct from the slaughter house.

GROWTH AT THE VARIOUS TEMPERATURES

The next step was to determine the temperature limits at which strain III would grow. Ten to 14°C. was arbitrarily selected as the lower limit. Strain III was inoculated on all three media and incubated at that temperature. On the peptic digest and Witte's peptone agar (hereafter to be designated as the "special" medium), and on plain agar, growth was visible in six days. In all experiments at this low temperature the eye could detect no difference in the rapidity or in the amount of growth on plain agar and on the special medium. After fifteen or twenty transplants growth was accelerated to such an extent that daily transplants could be made. The maximum amount of growth even at the end of six months was never more than 2 (using as before the same strain on plain agar at 37°C. as 10).

Considerable difficulty was experienced in obtaining any evidence of growth on the synthetic medium. At the start twenty-five transplants were made and carried at this temperature for five weeks before any growth was visible. All the tubes showing growth at that time (six) were again transplanted to the synthetic medium. From then on growth was fairly consistent though at any time a transfer might fail to grow and the method of inoculating several tubes from the previous transplant was resorted to. In this way the strain was accustomed to grow at a low temperature, though the maximum growth was never more than—1.

As the object of the work was to make the environmental changes as great as possible, it was decided to test the ability of strain III to grow at high temperatures without acclimatization. Growth was first tried at 45°C. and transplants on all three media were incubated at this temperature. At the end of twenty-four hours all tubes showed a growth almost as heavy

as on the corresponding medium at 37°C. The same procedure was repeated at 50°C. Growth was negative on the synthetic medium and but slight on the other two. Transplants of the special and plain agar cultures, however, failed to give any signs of growth.

The temperature was then reduced to 47°C. and the procedure repeated. Growth on the special and plain agar was very characteristic at this temperature. The first transplant grew fully as abundantly as did a control tube at 37°C. But as the number

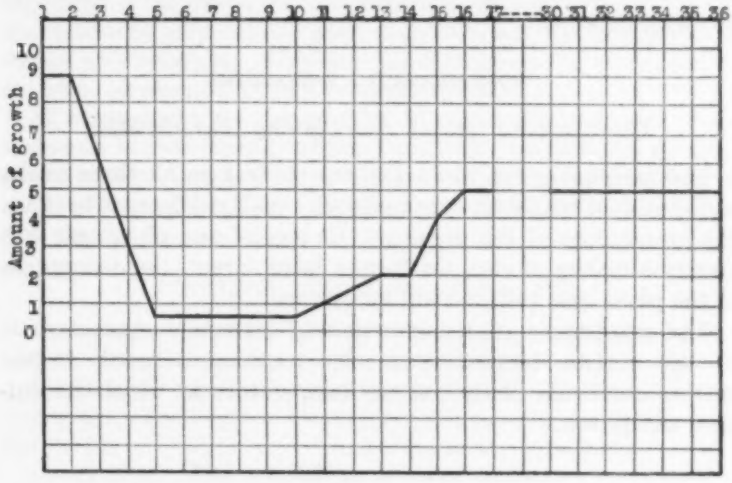

CHART 4

of transplants increased the amount of growth became less and less until at the fifth transplant the growth was barely perceptible. Then it increased again until it was equal to about one-half the growth on a control tube at 37°C. This curve will be seen on chart 4.

Strain III grew well on the first transplant to the synthetic medium. A second transfer showed slight growth in twenty-four hours, but a third transplant had to be incubated for five weeks in a sealed tube; when growth finally appeared in the form

of single colonies along the line of inoculation. After several generations a colony would give a uniform growth, though the maximum growth was never more than—1.

All ten strains were inoculated in the special medium and cultivated at 10° to 14°C. and at 47°C. Some variation was noted. All strains grew well on the special medium at the low temperature, but only four could be cultivated after the fifth transplant at 47°C. On the synthetic medium at both high and low temperatures strains II and VI grew much better than III. Strain X, though it grew well at 37°C., would not grow at either 10° to 14°C. or 47°C.

MORPHOLOGICAL VARIATIONS

Consistency of growth and staining characteristics

The morphology of strain III was studied on all three media at the various temperatures employed. As the difference between the morphology of the organisms on special and plain agar was merely a matter of size, the former being larger, the description of the plain agar cultures will be omitted.

The consistency of the growth was extremely characteristic at the various temperatures. By touching a needle to the culture one could easily tell the temperature at which the culture was grown.

SPECIAL MEDIUM AT 10° TO 14°C.

Consistency of growth. Moist and very viscid, long threads of the culture could be drawn from the tube by the needle. Considerable difficulty was experienced in emulsifying the culture in a drop of water on the slide. The organisms formed a scum on the surface of the water which came away with the needle. An extracellular substance was formed which resembled very closely that formed by the members of the capsulatus mucosus group. This substance often gave the effect of a capsule about the organisms.

Morphology. Rods from 20 to 30 micra long and 1 to 2 micra wide predominated in a fresh culture. Occasionally there were signs of terminal bulging. These swellings even after weeks of observation never became very pronounced. Frequently there were large elliptical,

extracellular globules about ten micra long and five micra wide which might possibly be the extracellular globules of Almquist (1908, 1911, 1922). As the age of the culture increased the size of the individual organisms decreased. A culture grown for three months showed bacilli morphologically indistinguishable from an agar control at 37°C.

Gram's stains. (Atkins' modification of this stain was used throughout these experiments.) A great difference was observed in the staining property of the bacteria. There was a marked tendency for all cultures on this medium to take the purple stain. Three or four minutes washing in alcohol or acetone was necessary to decolorize the smear. Clumps of organisms on the slide would frequently take the positive stain. Eighteen slides were prepared and given to an advanced class of thirty to be stained and examined. Forty-four per cent reported a Gram-positive rod while 56 reported a Gram-questionable rod.

All strains, with the exception of X, were cultivated under these conditions but the intensity of the reaction to Gram's stain was somewhat variable, though there was a tendency in all to give at least a questionable reaction. The consistency of growth and the morphology were the same in all.

SYNTHETIC MEDIUM AT 10° TO 14°C.

Consistency of growth. The same as with the special medium except that the extracellular substance was much less.

Morphology. Identical in all but one respect with the preceding description. The length of the rod was still about 20 to 30 micra but their width was reduced from 1 to 2 micra to about 0.5 micra.

Gram's stain. Clearly negative.

It therefore seems that the temperature rather than the composition of the medium determined the morphological variation. On the other hand the composition of the medium alone determined variation in Gram's stain. All cultures on being transplanted to plain agar and grown at 37°C. regained, after the third or fourth transplant, their normal morphological and staining characteristics.

SPECIAL MEDIUM AT 47°C.

Consistency of growth. A twenty-four hour culture is very moist and a loop is required to get any appreciable amount of the culture.

After several days the consistency of the growth is comparable to an agar control at 37°C.

Morphology. The actual length of the rod was scarcely more than its width. The longest form observed was only 4 micra long and 2 micra wide. The whole field was peculiar in that the organisms possessed no clearly defined outline. The general appearance resembled that of an amorphous precipitate more than it did bacteria. As the growth curve (chart 4) decreased this amorphous appearance became more and more pronounced. At the lowest point in the curve not a single typical rod could be found in the smear. As the curve rose again a more definite outline was observed but the organisms never regained their normal morphology. And it required fully twenty transplants on agar at 37°C. to bring back the normal form of the bacterium.

Gram's stain. Clearly negative, counter stain very faint.

SYNTHETIC MEDIUM AT 47°C.

Consistency of growth. Very watery, old cultures retaining their moisture even after the medium was dried to hardness.

Morphology. The variations in the morphology of the strain at this temperature are astounding. Practically every type of variation noted in the literature is represented in one smear from an acclimated culture. So different are the morphological changes at the various stages of growth that they can best be represented as follows:

1. Initial transplant: The amorphous forms described above. The shape of the individual bacilli was blurred but discernable.

2. Second transplant: All resemblance of definite form was lost. A few large oval bodies were present, which stood out in sharp contrast to the rest of the field. With the exception of the oval bodies the organisms stained very faintly.

3. Third transplant: After five weeks' incubation long thread forms began to appear. The oval bodies became fewer and swollen rods with definite outlines could be seen.

4. A fully acclimated culture: The predominating type was a rod about 20 to 30 micra long. At regular intervals along the rods were swollen portions about three times the normal width. There were many tennis racket forms in which the handle stained a light pink, while the head was bright red. A few of the deeply staining extracellular globules were to be seen in each field. The so called "dumb-bell" type was frequently met with. The terminal swellings stained deeply and the rest of the rod lightly. Invariably the swollen portion of any form stained heavily.

5. An old culture: This showed the same forms as an acclimated culture except that the swollen portions had become granular spots in the rod. The long rod looked very much like type B of the diphtheria bacillus (Wesbrooks classification). There were many faintly staining irregular masses that might be taken as the bacterial plasmodia described by Almquist. But in all my cultures I have never observed any bacterial microconidia. It may be possible that the intracellular granules mentioned above will eventually be cast out of the cell forming exogenous granules.

Before leaving the subject of culture media and its influence it would be well to mention a peculiar happening in the course of the experiments. The fourth lot of the special medium, made in exactly the same way, differed from all others in appearance. The organisms cultured at 37°C. did not grow as well on it as on previous lots. Under the microscope the bacilli appeared as typical lanceolate diplococcus forms. A culture suspended in salt solution to be examined for motility showed prompt and complete agglutination.

All ten strains were inoculated on this medium and examined for loss of fermentative powers and spontaneous agglutination. Not a single strain differed from the controls in the sugar broths. Strains I, III, IV, and X were promptly agglutinated in isotonic salt solution. Nor could they be suspended in any of the balanced solutions (Ringer's, Locke's, and Tyrode's). The use of balanced solutions in suspending spontaneously agglutinating, bacteria met with some success in the hands of Shearer (1919-1920) and Mellon (1922).

After several transplants on this medium the typhoid organism produced a golden yellow pigment. All strains behaved in the same manner. Another investigator working with the diphtheria bacillus found that this organism also produced a yellow pigment on the same medium. *Staphlococcus albus* grown on this medium was indistinguishable from *Staphlococcus aureus*. The method of making the medium was the same as in all other lots, so the peculiarity must have been in the livers or stomachs used in this particular case.

The medium was set aside for further investigation and was not used in the regular experiments.

IMMUNOLOGICAL VARIATIONS

1. Antigenic

Previous to any modification in the environment of strain III, which was used in the immunological work, its specificity was determined in immune sera produced by *Bact. coli; B. morgani; Bact. enteriditis; Bact. paratyphosum A; Bact. paratyphosum B;* and *Bact. dysenteriae.* There was no change in the specificity of the strain even after it had been cultivated for six months on the three media and at the temperatures previously described. All cultures used for immunization were carried, each in its particular environment, for five months before the first vaccine was made. For the purpose of avoiding any modifications in the antigenic properties of the bacteria due to heating or preservation, living vaccines were used. The vaccines were freshly prepared before each inoculation and standardized after the method of Hopkins.

Rabbits of the same weight, age and general health conditioms were selected. All were given the same diet and carefully observed to note any apparent variation in the toxicity of the cultures injected. Five inoculations were given, graded in the following manner:

First injection, 0.3 cc.
Second injection, 0.5 cc. six days after the first
Third injection, 0.8 cc. five days after the second
Fourth injection, 1.0 cc. five days after the third
Fifth injection, 1.0 cc. four days after the fourth

All rabbits used in the experiments were bled previous to any inoculation and a titration of the sera was made against the cultures to be used. The rabbits were inoculated with vaccines made from the following cultures:

Antigen			*Rabbit*
1	Special medium	47°C.	1
2	Plain agar	47°C.	2
3	Synthetic medium	47°C.	3
4	Special medium	37°C.	4
5	Plain agar	37°C.	5
6	Synthetic medium	37°C.	6

7	Special medium	10°C.	7
8	Plain agar	10°C.	8
9	Synthetic medium	10°C.	9

During the course of the immunization the animals were bled two days after the second, third, and fourth injections and the titer of the serum for its homologous antigen obtained. The final titer was obtained ten days after the last inoculation. The rise in the production of antibodies and the final titer of the sera from the nine rabbits are given in chart 5.

Chart 5 clearly shows that considerable alteration has taken place in the antigenic properties of the same strain cultivated under widely different circumstances. Strain III grown on the special medium at 37°C. produces a serum with a titer of 51,000 while the same strain grown on synthetic medium at 10° to 14°C. produces a serum whose titer is only 800.

INJECTIONS	RABBIT 1	RABBIT 2	RABBIT 3	RABBIT 4	RABBIT 5	RABBIT 6	RABBIT 7	RABBIT 8	RABBIT 9
2	400	400	100	1,600	800	800	200	200	100
3	3,200	800	400	25,600	1,600	3,200	800	1,600	400
4	6,400	3,200	1,600	25,600	3,200	12,800	3,200	6,400	200
X	25,600	6,400	12,800	51,200	12,800	25,600	6,400	6,400	800

CHART 5. TITER OF THE SERA FROM THE NINE RABBITS FOUR DAYS AFTER THE SECOND, THIRD AND FOURTH INJECTIONS
X = the final titer ten days after the fifth injection

It was hoped that a much closer relation could be shown between antigenic variation and the composition of the culture medium, but it will be noted that the synthetic medium at 37° and 47°C. gives a better antigen than plain agar at that temperature. In every case, however, the special medium produces a much better antigen than either the plain or synthetic agar.

2. Agglutinability

In order to show whether the antigenic alterations had been accompanied by any alteration in the agglutinating powers of the various cultures, the sera from the nine rabbits were titered against the nine antigens and the results tabulated in chart 6.

It will be readily seen from the chart that alterations in antigenic and agglutinating properties of strain III have run a fairly parallel course. The agglutinability of this strain grown on synthetic medium at low temperatures for five months decreased considerably since it agglutinates in only comparatively low dilutions in all nine sera. On the other hand a culture grown on the special medium at high temperatures shows a decided increase in its agglutinability, since for the most part it agglutinates in a heterologous serum in dilutions that are higher than those of homologous antigen and serum.

Strains II, VI and X were cultivated on synthetic agar at 37°C. for two months and tested for any alteration in their

ANTIGENS	SERUM 1	SERUM 2	SERUM 3	SERUM 4	SERUM 5	SERUM 6	SERUM 7	SERUM 8	SERUM 9
1	25,600	12,800	25,600	25,600	25,600	12,800	6,400	6,400	3,200
2	12,800	6,400	12,800	25,600	12,800	12,800	3,200	6,400	1,600
3	6,400	1,600	12,800	25,600	6,400	25,600	800	3,200	1,600
4	25,600	12,800	25,600	51,200	12,800	25,600	6,400	12,800	3,200
5	25,600	6,400	12,800	25,600	12,800	6,400	6,400	6,400	1,600
6	12,800	6,400	12,800	25,600	12,800	25,600	6,400	3,200	1,600
7	12,800	3,200	1,600	12,800	12,800	12,800	6,400	3,200	400
8	25,600	3,200	3,200	12,800	6,400	25,600	6,400	6,400	400
9	6,400	3,200	800	6,400	3,200	800	1,600	1,600	800

CHART 6. THE AGGLUTINABILITY OF THE ANTIGENS IN THE IMMUNE SERA

agglutinability with the serum from rabbit 5. Cultures of the same strains on plain agar at 37°C. were used as controls. Strains II and X showed some decrease in their agglutinability, while VI was comparable to the control in every way. Spontaneous agglutination of cultures grown on synthetic media was never encountered in any of these experiments.

PHYSIOLOGICAL REACTIONS

The physiological characteristics of all strains in all environments were remarkably constant. Cultures of strain III cultivated on the special medium, plain agar and synthetic medium at 47°, 37°, and 10° to 14° C were tested in their reaction in xylose, glucose, dextrin, manitol, glycerol, and lactose broths,

in gelatin, lead acetate and in the methyl red test. The results were in perfect accord with the results obtained in the same tests previous to any modifications in the environment of the strain (chart 1).

Strains II, VI and X were cultivated on synthetic agar at 37°C. and strains II and VI on the special medium at 10° to 14°C. for two months and tested as above. No variation, however, was noted in any case.

All ten strains were cultivated on plain agar containing immune serum and on malachite green agar. A characteristic loss in the agglutinability of all strains on both media was observed but their physiological reactions (tested as in the two foregoing experiments) remained unaltered.

It would seem from these experiments that in differentiating *Bact. typhosum* the physiological reactions, which remain unaltered under conditions that affect every other character of the species, are more to be relied upon than any other differential feature.

SUMMARY AND CONCLUSIONS

1. The power of growth of *Bacterium typhosum* is very different for different strains.

2. Morphological variation seems to be determined by both the composition of the medium and the temperature at which they are grown. The consistency of the growth is determined by the temperature alone.

3. Considerable variation in the immunological properties of a strain can be shown, but only when the antigens are cultivated under extremely favorable or unfavorable conditions.

4. All strains in all environments are remarkably constant in their physiological reactions.

5. The variations shown by a single strain, in any characteristic other than consistency of growth, cannot be considered as indicative for the whole species.

6. A variation, no matter how great, in any one character of a strain does not imply a variation in all the characters of that strain.

7. From a previous environment a strain may gain a certain momentum which for a short time will determine the character of its reaction in any new environment.

REFERENCES

ALMQUIST, E. 1908 Centralbl. f. Bakteriol., **48**, 175.
ALMQUIST, E. 1911 Centralbl. f. Bakteriol., **60**, 167.
ALMQUIST, E. 1922 Jour. Inf. Dis., **31**, 483.
AYRES, MUDGE AND RUPP 1920 Jour. Bact., **5**, 589.
DAMON, S. R. 1921 Jour. Biol. Chem., **48-49**, 379.
DOLT, L. 1908 Jour. Inf. Dis., **5**, 616.
DORYLAND 1916 Jour. Bact., **1**, 135.
EISENBERG 1906a Centralbl. f. Bakteriol., **41**, 83.
EISENBERG 1906b Centralbl. f. Bakteriol., **41**, 752.
FRANKEL AND BARBER 1919 Jour. Inf. Dis., **24**, 19.
HAMILTON, H. W. 1921 Jour. Bact., **6**, 43.
HERTER, C. A. 1909 Jour. Biol. Chem., **7**, 59.
JACOBY, MARTIN 1917 Biochem. Ztsch., **83**, 74.
JOACHIN 1904 Centralbl. f. Bakteriol., **36**, 662.
JOOS 1902 Centralbl. f. Bakteriol., **33**, 762.
KIRSTEIN 1904 Ztsch. f. Hyg. u. Infekt., **46**, 229.
MELLON, R. 1922 Jour. Med. Res., **53**, 345.
MORISHIMA 1920 Jour. Inf. Dis., **26**, 43.
NELSON, V. E. 1916 Jour. Biol. Chem., **24**, 527.
PACINIC AND RUSSELL 1918 Jour. Biol. Chem., **34**, 43.
PORGES AND PRANTSCHOFF 1906 Centralbl. f. Bakteriol., **41**, 752.
SCHELLER 1904 Centralbl. f. Bakteriol., **36**, 394.
SCHELLER 1905 Centralbl. f. Bakteriol., **38**, 100.
SEHRWALD 1905 Deutsch. Med. Wchnschr. **31**, 261.
SHEARER, C. 1919-20 Jour. Hyg., **18**, 337.
ZINSSER 1923 Infection and Resistance. New York.

THE DOCTOR'S FACTOTUM

August, 1924 Issue
Contains the following articles:

Food Protein Extracts For Desensitization

Food Desensitization In Bronchial Asthma

BACILLUS ACIDOPHILUS MILK
And How To Make It

What Is A Therapeutic Dose Of
BACILLUS ACIDOPHILUS?

POLLENS
Regional Distribution—Time And Method of Pollination

Copies available on request

THE ARLINGTON CHEMICAL CO.

DIFCO

MEDIA FOR THE ISOLATION OF INTESTINAL ORGANISMS

BACTO-ENDO'S AGAR

Bacto-Beef Extract	3 grams
Bacto-Peptone	5 grams
Bacto-Lactose	10 grams
Bacto-Agar	15 grams
Basic Fuchsin	0.4 gram
Sodium Sulfite	9 "

Dissolve 44 grams in 1000 c.c. distilled water and sterilize as usual.
Final pH = 7.5 ±.

BACTO-MACCONKEY'S AGAR

Bacto-Oxgall	5 grams
Bacto-Lactose	10 grams
Bacto-Agar	15 grams
Bacto-Peptone	20 grams
Neutral Red	0.05 gram

Dissolve 50 grams in 1000 c.c. distilled water and sterilize as usual.
Final pH = 7.0 ±.

BACTO-CONRADI-DRIGALSKI AGAR

Bacto-Peptone	10 grams
Sodium Caseinate, Difco	10 grams
Bacto-Lactose	10 grams
Bacto-Agar	15 grams
Sodium Chloride C.P.	5 grams
Basic Crystal Purple	0.03 gram
Crystal Violet	0.02 gram

Dissolve 50 grams in 1000 c.c. distilled water and sterilize as usual.
Final pH = 6.8 ±.

Specify "DIFCO"

THE TRADE NAME OF THE FINEST

In the research and development of Bacto-Peptone and Dehydrated Culture Media

DIGESTIVE FERMENTS COMPANY

DETROIT, MICHIGAN, U. S. A.